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## GRAHAM LUSK

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The Journal records with deep regret the death of Graham Lusk on July 18, 1932, at the age of 66 years. Professor Lusk was for many years, and up to the time of his death, President of The Journal of Biological Chemistry, Inc., and a member of the Editorial Committee of the Journal. The loss of this colleague who has, during a period of more than forty years, exemplified in his career the finest traditions of research and the highest ideals of academic life will be keenly felt wherever the physiological sciences are recognized.

After the completion of an undergraduate course at the School of Mines of Columbia University, which afforded him an excellent training in chemistry, Lusk proceeded to Munich to begin the advanced study of physiology under the guidance of Carl von Voit. There he received the degree of Doctor of Philosophy from the University. The years spent in the environment of this vigorous teacher left an indelible impression upon the young pupil; and he delighted on all suitable occasions to refer with characteristic enthusiasm and reverence to Voit's influence and contributions to the science of physiology.

Dr. Lusk began his independent career as a teacher of physiology and an investigator in the field of nutrition in 1891 at the Medical School of Yale University where he was rapidly advanced to the rank of a full professor. In 1895 he joined the staff of the University and Bellevue Hospital Medical College, New York City,—an institution with which his distinguished father, Dr. William T. Lusk, had been affiliated. In 1909 Dr. Graham Lusk became Professor of Physiology at Cornell University Medical College in New York City, a post from which he retired in June, 1932. For many years he served as Scientific Director of the Russell Sage Institute of Pathology. He was active in the formation and support of the Harvey Society and the Society for Experimental Biology and Medicine.

Those who knew Dr. Lusk most intimately will cherish the memory not only of what he achieved for science and education but also of a lovable and charming personality. To those who were close to him, his friendship was a priceless treasure.





# MAXIMUM ROTATIONS AND CORRELATION OF DISUBSTITUTED ACETIC ACIDS CONTAINING A METHYL GROUP

By P. A. LEVENE AND R. E. MARKER

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

(Received for publication, June 20, 1932)

In a recent article<sup>1</sup> the rotations of members of homologous series of disubstituted propionic, butyric, and valeric acids were compared (each containing the substituting alkyl groups on the terminal carbon atom, and one of the groups attached to the asymmetric carbon atom being a methyl group). The starting member was levo-3-methyl-3-ethylpropionic acid. The values of the rotations of the members of the homologous acids progressively increased towards the right. Similar changes were observed in the members of the derivatives of the configurationally related butyric and valeric acids and in the configurationally related propyl carbinols and propyl chlorides derived from the homologous members of the levo-3-methyl-3-ethylpropionic acids. These observations seemed to point to the possibility of formulating a general conclusion to the effect that the direction of the change in rotations of consecutive members is the same for all configurationally related homologous series. At the time of that publication<sup>1</sup> the maximum values of the rotations of the members of the homologous series of the corresponding substituted acetic acids were not yet known. Hence, several members of this series have been resolved until further increase in their rotation was not obtainable by the methods available at present. The results are given in Table I, in which, for comparison, the rotations of the corresponding members of the acids previously studied are also given.

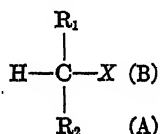
From Table I it may be seen that the values of the rotation of

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 153 (1932).

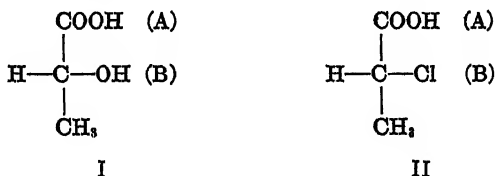
TABLE I  
Maximum Molecular Rotations of Configurationally Related Aliphatic Acids Containing a Methyl Group on the Asymmetric Carbon Atom,  $[M]_D^{25}$  (Homogeneous)

$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$	-18.0	-10.4	-13.6	-11.1	-12.2
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$	-21.4	+3.6	-6.9	-3.7	
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COOH} \\   \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_4\text{H}_9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_4\text{H}_9 \end{array}$	-24.3	+6.1	-4.1	-1.7	
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_7\text{H}_{15} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COOH} \\   \\ \text{C}_7\text{H}_{15} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_7\text{H}_{15} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_7\text{H}_{15} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_7\text{H}_{15} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\   \\ \text{C}_7\text{H}_{15} \end{array}$	-27.3	+8.1	-1.9	-0.6	
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_{10}\text{H}_{21} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COOH} \\   \\ \text{C}_{10}\text{H}_{21} \end{array}$					-27.5				

the members of the substituted acetic acid series progressively increase to the left, contrary to the conduct of the members of the other series. Thus the rule of the correlation of the configurations of homologous series by the direction of the change of rotation of consecutive members is limited to those series in which the functional group is attached to a carbon atom located at a distance from the asymmetric carbon atom. In fact, the older observations on the  $\alpha$ -thiol and  $\alpha$ -sulfo acids already pointed towards the possibility that in two configurationally related homologous series,<sup>1</sup> the change in rotation of consecutive members may have a different direction when the series differ in the character of a functional group attached directly to the asymmetric carbon atom. From theoretical considerations, such a state of affairs is readily understandable. If, as in the previous communication, the contributions of the two groups are designated by the letters A and B,



and if it is assumed that in consecutive members both values increase numerically, then in the visible part of the spectrum the change of the direction of rotation of the consecutive members will depend upon whether A or B furnishes the higher contribution. Thus, if we take the case of two acids



and assume that the numerical values of each increase in consecutive members, then the direction of the change will depend upon whether A or B furnishes the major contribution. Thus, assuming that in both I and II the  $-COOH$  group has a dextrorotatory and B a levorotatory contribution and that in I the major contribution is furnished by A, and in II by B, the changes in I may be to the right and in II to the left.

In addition to the data on the maximum rotation of the members of the disubstituted acetic acids, the present article contains additional evidence towards the correlation of the configurations of the series of disubstituted acetic and propionic acids.

TABLE II

*Correlation of Methyl-n-Propylpropionic Acid to Methyl-n-Propylacetic Acid,  $[M]_D^{25}$  (Homogeneous) (Not Maximum Rotations)*

$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COOH} \\   \\ \text{C}_3\text{H}_7 (n) \\ +1.0^* \end{array}$	→	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{COCl} \\   \\ \text{C}_3\text{H}_7 (n) \\ -1.3 \end{array}$	→	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{CONH}_2 \\   \\ \text{C}_3\text{H}_7 (n) \\ +1.9 \end{array}$	↘	$\begin{array}{c} +4.1 \\ \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2\text{NH}_2 \\   \\ \text{C}_3\text{H}_7 (n) \\ +3.7^\dagger \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_3\text{H}_7 (n) \\ -6.8 \end{array}$	→	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COCl} \\   \\ \text{C}_3\text{H}_7 (n) \\ -4.6 \end{array}$	→	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CONH}_2 \\   \\ \text{C}_3\text{H}_7 (n) \\ -6.7^\ddagger \end{array}$	→	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CN} \\   \\ \text{C}_3\text{H}_7 (n) \\ -13.4 \end{array}$

\* For intermediate steps, see Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 91, 77 (1931).

† Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 84, 571 (1929).

‡ In alcohol.

The evidence thus far furnished is the following: (1) 3-Methyl-3-ethylpropionic acid has been correlated with the corresponding acetic acid by the synthesis of 4-methyl-4-ethylbutyric acid from 2-methyl-2-ethyl-1-bromoethane to which then was correlated 3-methyl-3-ethylpropionic acid. (2) 3-Methyl-3-*n*-butylpropionic acid has been correlated with the corresponding acetic acid by

the 2-methyl-2-*n*-butyl-1-aminoethanes derived from them. (3) 3-Methyl-3-*n*-propylpropionic acid has now been correlated with the acetic acid series by the set of reactions given in Table II.

Thus, three members of the acetic acid series have been correlated by direct chemical methods with the corresponding members of the propionic acid series. Hence, it is justified to conclude that the higher members of the two series given in the first and second columns of Table I also are configurationally related. It thus follows that the increase in rotation of the consecutive members follows a different direction in each of these two series. Work is now in progress to determine which group in each series is responsible for the direction of rotation in the visible part of the spectrum.

#### EXPERIMENTAL

*Methylethylacetic Acid*—The inactive acid was prepared by the malonic ester synthesis. This was resolved by means of its brucine salt from water as described by Marckwald.<sup>2</sup> The maximum value of the rotation was the same as that reported by him,  $[M]_D^{25} = +18^\circ$  (homogeneous).

*Levo-Methyl-*n*-Propylacetic Acid*—The inactive acid was dissolved in boiling acetone and an equivalent weight of quinine was added. After eighteen recrystallizations, the rotation of the free acid reached a constant value. B.p.  $96^\circ$  at 15 mm.  $D_{\frac{25}{4}} = 0.920$ .  $n_D^{25} = 1.4117$ .

$$[\alpha]_D^{25} = \frac{-16.90^\circ}{1 \times 0.920} = -18.4^\circ; [M]_D^{25} = -21.4^\circ \text{ (homogeneous)}$$

4.025 mg. substance: 9.200 mg. CO<sub>2</sub> and 3.835 mg. H<sub>2</sub>O

C<sub>6</sub>H<sub>12</sub>O<sub>2</sub>. Calculated. C 62.0, H 10.4

116.1 Found. " 62.3, " 10.7

0.063 gm. substance: 5.45 cc. 0.1 N NaOH (titration, phenolphthalein). Calculated, 5.43 cc.

*Levo-Methyl-*n*-Butylacetic Acid*—The inactive acid was dissolved in hot 66 per cent acetone and an equivalent weight of cinchonidine added. After eight recrystallizations from 66 per cent acetone

<sup>2</sup> Marckwald, W., *Ber. chem. Ges.*, **37**, 1045, 1048 (1904).

the rotation of the free acid reached a constant value. B.p.  $105^{\circ}$  at 5 mm.  $D_{\frac{25}{4}} = 0.909$ .  $n_D^{25} = 1.4189$ .

$$[\alpha]_D^{25} = \frac{-17.0^{\circ}}{1 \times 0.909} = -18.7^{\circ}; [M]_D^{25} = -24.3^{\circ} \text{ (homogeneous)}$$

3.980 mg. substance: 9.490 mg.  $\text{CO}_2$  and 3.920 mg.  $\text{H}_2\text{O}$

$\text{C}_7\text{H}_{14}\text{O}_2$ . Calculated. C 64.6, H 10.8

130.1 Found. " 65.0, " 11.0

0.1030 gm. substance: 7.86 cc. 0.1 N NaOH (titration, phenolphthalein).  
Calculated, 7.92 cc.

*Levo-Methyl-n-Heptylacetic Acid*—The inactive acid was dissolved in boiling acetone and an equivalent weight of cinchonidine was added. After twelve recrystallizations the rotation of the free acid reached a constant value. B.p.  $115^{\circ}$  at 1 mm.  $D_{\frac{25}{4}} = 0.895$ .  $n_D^{25} = 1.4312$ .

$$[\alpha]_D^{25} = \frac{-14.25^{\circ}}{1 \times 0.895} = -15.9^{\circ}; [M]_D^{25} = -27.3^{\circ} \text{ (homogeneous)}$$

5.430 mg. substance: 14.020 mg.  $\text{CO}_2$  and 5.770 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{20}\text{O}_2$ . Calculated. C 69.7, H 11.6

172.16 Found. " 70.4, " 11.8

0.146 gm. substance: 8.55 cc. 0.1 N NaOH (titration, phenolphthalein).  
Calculated, 8.49 cc.

*Dextro-Methyl-n-Decylacetic Acid*—The inactive acid was dissolved in boiling acetone and an equivalent weight of cinchonidine was added. It was allowed to crystallize in a refrigerator at  $-10^{\circ}$ . After ten recrystallizations there was no change in the rotation of the free acid. The total was treated with hydrochloric acid, extracted with ether, purified through its sodium salt, then distilled. B.p.  $150^{\circ}$  at 1 mm.  $D_{\frac{25}{4}} = 0.884$ .  $n_D^{25} = 1.4395$ .

$$[\alpha]_D^{25} = \frac{+11.35^{\circ}}{1 \times 0.884} = +12.85^{\circ}; [M]_D^{25} = +27.5^{\circ} \text{ (homogeneous)}$$

4.116 mg. substance: 11.085 mg.  $\text{CO}_2$  and 4.555 mg.  $\text{H}_2\text{O}$

$\text{C}_{13}\text{H}_{26}\text{O}_2$ . Calculated. C 72.8, H 12.2

214.2 Found. " 73.4, " 12.4

0.137 gm. substance: 6.46 cc. 0.1 N NaOH (titration, phenolphthalein).  
Calculated, 6.40 cc.

*Dextro-2-Methyl-1-Aminopentane*—25 gm. of 2-propylbutyric acid-4,  $[\text{M}]_D^{25} = +1.01^\circ$  (homogeneous), were converted into the amide as previously described.<sup>3</sup>

18 gm. of bromine were dissolved in 60 gm. of potassium hydroxide in 1 liter of water. This solution was poured onto 20 gm. of amide. The mixture was heated on the steam bath for 1 hour, then steam-distilled. The distillate was acidified with hydrochloric acid, extracted with ether, the concentrate then made alkaline, and the amine extracted with ether. B.p.  $64^\circ$  at 90 mm.  $D_{\frac{25}{4}} = 0.763$ .

$$[\alpha]_D^{25} = \frac{+3.12^\circ}{1 \times 0.763} = +4.09^\circ; [\text{M}]_D^{25} = +4.13^\circ \text{ (homogeneous)}$$

3.280 mg. substance: 8.550 mg.  $\text{CO}_2$  and 4.395 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{18}\text{N}$ .	Calculated.	C 71.2,	H 14.9
101.12	Found.	" 71.1,	" 15.0

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<sup>3</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).





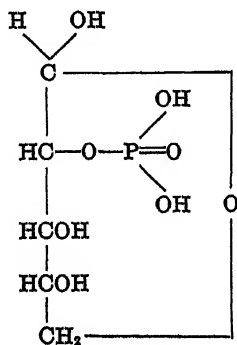
# THE RIBOSEPHOSPHORIC ACID FROM XANTHYLIC ACID. II

BY P. A. LEVENE AND STANTON A. HARRIS

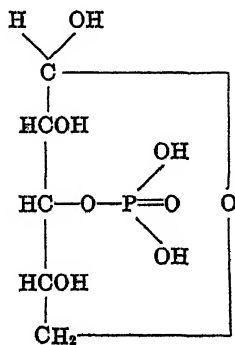
(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

(Received for publication, June 20, 1932)

In a previous communication<sup>1</sup> it was shown that the ribosephosphoric acid obtained from xanthylic acid and hence from guanylic acid was different from 5-ribosephosphoric acid derived from inosinic acid. Furthermore, it was found by Levene and Tipson<sup>2</sup> that guanosine and therefore xanthosine possess the furanoside structure. Thus only two structures should be considered for the new ribosephosphoric acid; namely, the one with the phosphoric acid residue in position (2) and that in position (3). In the present communication conclusive evidence is furnished in favor of the latter structure (II).



I



II

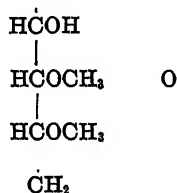
The methylation method was first chosen for the purpose of establishing the structure of the new ribosephosphoric acid. After

<sup>1</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **95**, 755 (1932).

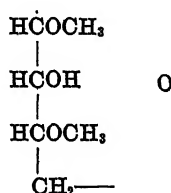
<sup>2</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **97**, 491 (1932).

the methylation was accomplished, two alternative methods *a priori* were available for establishing the position of the phosphoric acid.

The first method was the removal of the phosphoric acid with subsequent isolation of the methylated normal riboside. The structure of the dimethylriboside, depending upon the position of the phosphoric acid residue, should be either III or IV. Only substance III can yield an optically active dimethyltrihydroxyglutaric acid.

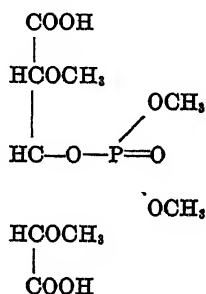
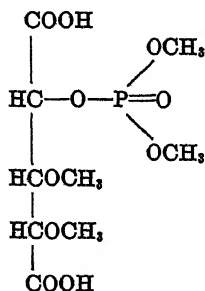


III



IV

The second method was the oxidation of the methylated ribosephosphoric acid to the corresponding phosphorylated trihydroxyglutaric acid. Only one (V) of the two possible acids could be optically active.

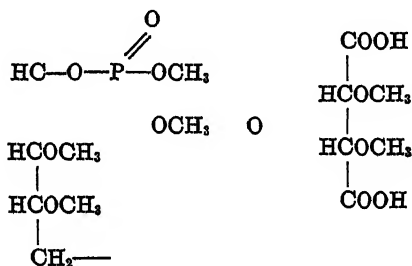


VI

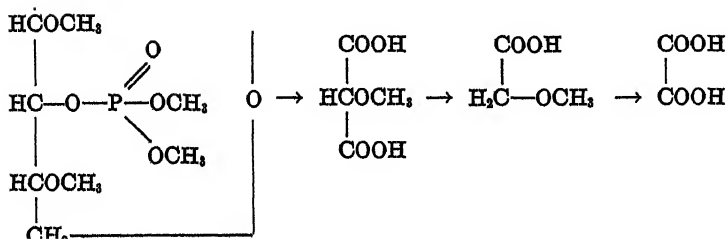
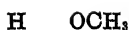
However, neither of these two methods yielded satisfactory results. The failure of the first was due to the unexpected resist-

ance of the phosphoric acid residue towards the hydrolytic action of hydrogen or hydroxyl ions, so that it was not possible to obtain a satisfactory yield of the methylated sugar.

The second method was also found unsatisfactory. By the milder oxidation with nitric acid, the expected dimethyltrihydroxyglutaric acid was not obtained. More rigorous oxidation was then attempted. It was expected that this procedure might lead to simultaneous dephosphorylation and oxidation of the dimethylribose. Either mesodimethyltartaric acid, in case of VII, or methoxymalonic acid, in case of VIII, should be formed. The



VII



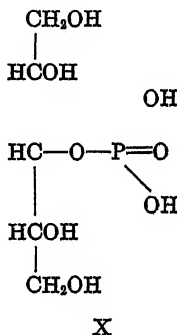
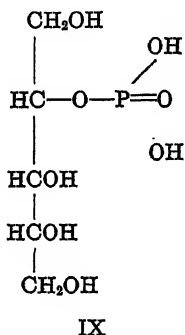
VIII

latter substance would be expected to form methylglycolic acid, which perhaps could be oxidized into oxalic acid. Indeed, oxalic acid was the only substance which could be identified among the

products of the reaction. Thus, by this method indirect evidence was secured in support of II. The evidence, however, was not conclusive.

In view of the unsatisfactory results obtained by both of these methods, it was decided to resort to the reduction of the *d*-ribosephosphoric acid to the corresponding *d*-ribitolphosphoric acid. Again one of two alternative possibilities was envisaged, depending upon the structure of the new ribosephosphoric acid.

Substance I should lead to an optically active ribitolphosphoric acid, IX, whereas substance II should form an optically inactive derivative, X.



Two methods available for the reaction were: the reduction by means of sodium amalgam and the catalytic reduction by hydrogen in the presence of platinic oxide (reagent of Roger Adams). In a preliminary way, both methods were tested on hexose-3-phosphate. In this case more satisfactory results were obtained by the amalgam reduction. On the other hand, in the case of *d*-ribosephosphoric acid, the catalytic method gave a better yield of a purer material which proved to be the inactive ribitolphosphoric acid which consequently had the structure X. The optical activity was tested both in aqueous solution and in a solution of sodium borate. Thus, the structure of the new ribosephosphoric acid is definitely established as *d*-ribose-3-phosphoric acid (II).

#### EXPERIMENTAL

*Methylation of Ribosephosphoric Acid*—A solution containing 25 gm. of barium ribosephosphate was treated with dilute sulfuric

acid until no test for barium or sulfate was obtainable. This solution was then shaken with a slight excess of silver oxide (16.6 gm.). The silver salt was precipitated by adding an equal volume of methyl alcohol. The precipitate was washed with methyl alcohol and ether and dried to constant weight over phosphorus pentoxide in a vacuum desiccator. The yield of silver salt was 22.5 gm. or 72 per cent of the theoretical.

25 gm. of the dry silver salt were treated with 960 cc. of reagent methyl alcohol containing 8.5 gm. of hydrogen chloride. This gave a solution of ribosephosphoric acid containing approximately 0.5 per cent of hydrogen chloride. This solution was allowed to stand for 9 days at 50° and 4 days at 58°, after which it was filtered and neutralized with dry silver oxide. No appreciable amount of phosphoric acid was liberated at 50°, but after 4 additional days at 58°, it was found that 17 per cent of the phosphorus had been hydrolyzed. Enough silver oxide was used to form the silver salt of the phospho-*n*-methylriboside. The solution containing the precipitated silver salt was evaporated to one-half its volume. It was then treated with a total of 23 cc. of methyl iodide and heated on a steam bath for 2 hours. The silver halides were removed by filtration and thoroughly washed with methyl alcohol. The filtrate, containing the methyl ester of the phospho-*n*-methylriboside, was evaporated to a thick syrup. This syrup was taken up in 35 cc. of methyl iodide and 15 cc. of methyl alcohol. Methylation started spontaneously on the addition of silver oxide. The material was methylated and remethylated once by the Purdie process.

After completion of the methylation the silver oxide and silver iodide were filtered off and extracted five times with boiling chloroform. The chloroform solution was dried over sodium sulfate and concentrated to a syrup under reduced pressure. On distillation a forerun was obtained, distilling at 65° at 0.5 mm. pressure and weighing 3.5 gm. Judging from analytical data, it consisted of methylated phosphoric acid and methylated ribose. The remainder distilled at 128–133° under 0.1 mm. pressure. The yield was 5.1 gm. The refractive index of the methylated product from the higher boiling fraction was  $n_D^{25} = 1.4498$ .

The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-1.765^\circ \times 100}{5.13 \times 2} = -17.2^\circ$$

The substance had the composition of dimethylphosphodimethyl-*n*-methylriboside.

5.480 mg. substance:	8.109 mg. CO <sub>2</sub> and 3.520 mg. H <sub>2</sub> O
5.554 " "	21.800 " AgI
4.372 " "	31.240 " ammonium phosphomolybdate
C <sub>16</sub> H <sub>21</sub> O <sub>8</sub> P. Calculated.	C 40.00, H 7.00, OCH <sub>3</sub> 51.6, P 10.32
Found.	" 40.35, " 7.19, " 51.81, " 10.37

### *Dephosphorylation Experiments*

Two 0.1 gm. samples of the fully methylated phosphoribose were dissolved in 25 cc. of water containing respectively 2 molecular equivalents of hydrochloric acid and sodium hydroxide. Small portions were sealed in test-tubes and heated in an oil bath at 140°. At stated intervals the amount of free phosphorus was determined by the Kuttner-Cohen<sup>3</sup> method. After 2½ hours the acid solution was found to be 99 per cent hydrolyzed, while the basic solution liberated only 12 per cent of its phosphorus in 16 hours. Only 50 per cent of free sugar was found by the Hagedorn-Jensen<sup>4</sup> titration. At steam bath temperature the rate of hydrolysis with 0.1 N HCl was very much slower. Hydrolysis in ammonium acetate solution at 140° showed less than 5 per cent free phosphorus. An attempted hydrolysis of 2 gm. of material in a sealed tube at 140–150° showed only 70 per cent liberation of free phosphorus.

*Oxidation of the New Ribosephosphoric Acid by Means of Nitric Acid*—The procedure was essentially that of Levene and Tipson.<sup>5</sup> After the removal of the nitric acid by distillation, the residue was transferred to a distilling flask with acetone. The acetone was replaced with methyl alcohol. After evaporation to a syrup, it was dissolved in methyl alcohol containing 2 per cent of hydrogen chloride. This solution was refluxed for 1 hour. The methyl alcohol was removed by distillation and the distillate was boiled with an excess of barium hydroxide. A precipitate was formed which was proved, by qualitative tests, to be barium oxalate. The residue left after the distillation of the methyl alcohol

<sup>3</sup> Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927). See also Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928).

<sup>4</sup> Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923). See also Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

<sup>5</sup> Levene, P. A., and Tipson, R. S., *J. Chem. Soc.*, **93**, 623 (1931).

was thoroughly extracted with ether. No appreciable amount of material was found in the ether extract. The methyl ester of dimethyltartaric acid would have been found here if any had been formed.

The ether-insoluble residues contained an acid or acids with a very low phosphorus content. The only positive result from this experiment was the isolation of oxalic acid as the barium salt.

*Reduction with Sodium Amalgam*—The experiment was first tried with barium hexose-3-phosphate. Catalytic reduction proceeded very slowly and so reduction with sodium amalgam was attempted. The Fehling's test was practically negative after 50 to 60 hours and the Hagedorn-Jensen titration showed 97 per cent reduction. Very little phosphorus was liberated in this process. The same method was tried with barium ribosephosphate. Only 2 gm. of product were isolated from 10 gm. of starting material. The material was not very pure as shown by its analysis. The substance exhibited a slight optical activity.

*Catalytic Reduction*—5 gm. of barium ribosephosphate were dissolved in 25 cc. of water to which 0.5 gm. of platinum oxide catalyst was added. This mixture was shaken with hydrogen at 40 pounds pressure for 20 hours.

A Hagedorn-Jensen titration showed that 98 per cent reduction had taken place. The catalyst was filtered off, and the solution diluted, filtered, and concentrated to a volume of 10 to 15 cc. The salt was precipitated by pouring into absolute alcohol. It was purified by dissolving in a small amount of water, making slightly alkaline with barium hydroxide, and reprecipitating with absolute alcohol. Analysis showed that it had the constitution of barium ribitolphosphate.

4.500 mg. substance:	2.660 mg. CO <sub>2</sub> and 1.300 mg. H <sub>2</sub> O
4.620 " "	26.20 " ammonium phosphomolybdate
0.1000 gm. "	0.0620 gm. BaSO <sub>4</sub>
C <sub>8</sub> H <sub>11</sub> O <sub>8</sub> PBa. Calculated.	C 16.33, H 2.99, P 8.44, Ba 37.40
Found.	" 16.11, " 3.23, " 8.23, " 36.49

The rotation was taken in the following manner. Exactly 0.5 gm. of barium ribitolphosphate was dissolved in 1 to 2 cc. of water and treated with an exact equivalent of sodium oxalate. The barium oxalate was filtered, washed, and the filtrate evaporated



to a small volume. This was filtered, diluted to 10 cc., and the optical rotation taken in a 2 dm. tube. The reading was  $0^\circ$ . 5 cc. of this solution were diluted with 5 cc. of saturated borax solution ( $25^\circ$ ) and the optical rotation was again taken. The reading was less than  $+0.01^\circ$  ( $\pm 0.02^\circ$ ).

The same procedure with the same quantities was carried out with barium ribosephosphate. The rotation calculated on the basis of sodium ribosephosphate is given below.

$$[\alpha]_D^{25} = \frac{-0.67^\circ \times 100}{3.749 \times 2} = -8.93^\circ \text{ (in water)}$$

When 5 cc. of this solution were diluted with 5 cc. of borax solution, the reading was

$$[\alpha]_D^{25} = \frac{+1.53^\circ \times 100}{3.749 \times 2} = +40.81^\circ$$

# A NOTE ON THE USE OF 1-BROMOTETRAMETHYL- GLUCOSE FOR THE SYNTHESIS OF METHYLATED GLYCOSIDES

By P. A. LEVENE AND FRANK CORTESE

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

(Received for publication, July 12, 1932)

When synthesis is undertaken for the purpose of establishing the structure of a substance, the task is accomplished equally well if the synthesis of a known derivative of the substance is attained. Inasmuch as modern methods have made it feasible to methylate practically all glycosides, it is sufficient for structural purposes to be able to synthesize the methylated glycosides. The advantages which these offer are many and one of the most important is that they are volatilizable. Thus far only few similar syntheses are recorded; namely, those of Freudenberg and his collaborators<sup>1</sup> who employed for this purpose 1-chlorotetramethylglucose and 1-chloroheptamethylcellobiose. We thought that 1-bromotetramethylglucose which is more accessible than the chloro derivative might serve the purpose to advantage.

In this note the method of preparation of 1-bromotetramethylglucose is reported and in a preliminary way its conversion into tetramethylmethylglucopyranoside and into theophylline-tetramethylglucopyranoside. The details of preparation are given in the experimental part. Still better results than those reported undoubtedly will be obtained when larger quantities of material will be used so as to permit further purification.

## EXPERIMENTAL

*Acetylation of Tetramethylglucose*—40 gm. of tetramethylglucose were dissolved in 200 cc. of acetic anhydride. 15 gm. of freshly

<sup>1</sup>Freudenberg, K., Andersen, C. C., Go, Y., Friederich, K., and Richtermyer, N. W., *Ber. chem. Ges.*, **63**, 1964 (1930).

fused sodium acetate were added and the mixture was refluxed 10 minutes. After cooling, 200 cc. of dry ether and 300 cc. of toluene were added and the whole was evaporated to a syrup under diminished pressure in a bath at 50°. This evaporation was repeated twice with two portions of toluene (200 cc.). The product was dissolved in dry ether, the sodium acetate filtered off, and the filtrate evaporated to a syrup which was distilled with an oil pump. The fraction boiling at 133–138° was collected. The yield of thick colorless and odorless oil amounted to 37.3 gm.

The substance had the following composition.

3.665 mg. substance: 7.00 mg. CO<sub>2</sub> and 2.570 mg. H<sub>2</sub>O

3.291 " " : 10.290 " AgI

C<sub>12</sub>H<sub>22</sub>O<sub>7</sub>. Calculated. C 51.80, H 7.91, OCH<sub>3</sub> 44.60

Found. " 52.08, " 7.84, " 41.27

0.1644 gm. substance required 5.91 cc. 0.1 N NaOH

C<sub>12</sub>H<sub>22</sub>O<sub>7</sub>. Calculated, CH<sub>3</sub>CO 15.46; found, CH<sub>3</sub>CO 15.46

*1-Bromo-2,3,4,6-Tetramethylglucose*—10 gm. of tetramethylglucose acetate were dissolved in a solution of 60 gm. of hydrogen bromide in 60 gm. of glacial acetic acid. The reaction mixture was kept in a bath of ice water for 1 hour, after which the solution was diluted with large amounts of dry ether and toluene and concentrated to a syrup under diminished pressure in a bath at 50°. This evaporation was repeated twice with two 200 cc. portions of toluene. The residual syrup amounted to 10 gm. and was initially light yellow but soon changed to a deep red. In fact, the compound is very unstable.

The substance had the following composition.

0.1120 gm. required 3.38 cc. 0.1 N AgNO<sub>3</sub>

C<sub>10</sub>H<sub>18</sub>O<sub>5</sub>Br. Calculated, Br 26.85; found, Br 24.32

*Methylation of 1-Bromo-2,3,4,6-Tetramethylglucose*—10 gm. of freshly prepared bromotetramethylglucose were treated with 50 cc. of dry methyl alcohol and 12 gm. of dry silver carbonate. Charcoal was added and the mixture was filtered. After evaporating the alcohol under diminished pressure, the residual syrup was distilled with an oil pump. 4 cc. distilled over and about 3 cc. remained as a deep red viscid tar. The distillate was fractionated at high vacuum and was shown to be one-third tetramethyl- and two-thirds pentamethylglucose.

The substances had the following composition.

*Fraction I*

4.686 mg. substance: 9.094 mg. CO<sub>2</sub> and 3.610 mg. H<sub>2</sub>O

3.865 " " : 17.06 " AgI

C<sub>11</sub>H<sub>22</sub>O<sub>6</sub>. Calculated. C 52.80, H 8.80, OCH<sub>3</sub> 62.00

Found. " 52.92, " 8.62, " 59.04

*Fraction II*

3.988 mg. substance: 7.530 mg. CO<sub>2</sub> and 2.960 mg. H<sub>2</sub>O

3.753 " " : 14.152 " AgI

C<sub>10</sub>H<sub>20</sub>O<sub>6</sub>. Calculated. C 50.85, H 8.47, OCH<sub>3</sub> 52.5

Found. " 51.49, " 8.30, " 49.8

*Theophylline-2,3,4,6-Tetramethylglucoside*—15 gm. of dry silver theophylline were added to a solution of 10 gm. of 1-bromo-2,3,4,6-tetramethylglucose in 190 cc. of dry xylene and the mixture boiled for 45 minutes under a reflux. After cooling, the solution was filtered into 1500 cc. of petroleum ether. This was then filtered and the liquid concentrated under diminished pressure. The residue, consisting of 6 cc. of a yellow viscid gum, was distilled at 0.006 mm. to give the following fractions.

Bath	Fraction No.	Amount	Description
°C.		cc.	
120-140	I	1.6	Colorless and mobile
140-180	II	1.0	Very viscous, deep yellow gum
180-220	III	2.0	Solidified on cooling into deep yellow transparent solid
	Residue	1.0	Deep red transparent solid

*Fraction III*—This was redistilled and had the following composition.

3.676 mg. substance: 0.406 mg. N<sub>2</sub> (25° and 738 mm.)

C<sub>17</sub>H<sub>28</sub>O<sub>7</sub>N<sub>4</sub>. Calculated, N 13.22; found, N 12.28

The glucoside did not reduce Fehling's solution until after acid hydrolysis.



## THE EFFECT OF DIETARY DEFICIENCIES ON PHOSPHOLIPID METABOLISM\*

By BETTY R. MONAGHAN†

(From the Department of Zoology, Washington University, St. Louis)

(Received for publication, June 20, 1932)

The starting point for the present investigation was the observation, by Monaghan and Schmitt (1932), that vitamin A, and to a lesser extent its precursor, carotene, inhibit the oxidation of unsaturated fatty acids *in vitro*. The number of compounds known to exert such a protective action are distinctly limited (Mattill, 1931), and with few exceptions, do not occur in living organisms. Considering the importance of these acids to the animal organism (Burr and Burr, 1930) and the ease with which they normally undergo oxidation in the pure state, this property of vitamin A became especially significant. The possibility that the normal utilization of these acids in the organism may depend upon the presence of this vitamin in the diet was immediately suggested. The data herein presented were obtained in an effort to put this possibility to the test.

The first step was to determine, if possible, from a consideration of the known facts concerning fat metabolism and the action of vitamin A, what particular step in the utilization of the unsaturated fatty acids might be expected to require this vitamin. It has been clearly demonstrated by the work of Drummond (1919) that vitamin A is not required for the absorption of fatty acids from the intestine, or for the synthesis of the absorbed acids into neutral fats. It seems equally clear that this vitamin is not essential for the conversion of carbohydrate into fat, nor for the desaturation of fatty acids, since it has been shown repeatedly that the

\* The material presented in this paper is part of a thesis submitted to Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow in Zoology.

feeding of large amounts of highly unsaturated fat neither prevents nor cures the typical symptoms of vitamin A deficiency (provided, of course, the fat is free from vitamin). Furthermore, it is well known that fatty acids more unsaturated than oleic, whether derived from the food or manufactured in the body, are utilized mainly in the formation of phospholipids rather than in the synthesis of neutral fat. The depot fat of warm blooded animals, and the neutral fat of the tissues, except in the case of the liver, generally contain fatty acids that are considerably more saturated than those of the phospholipids (Bloor, 1926, 1927, 1928).

On the basis of these considerations, the most promising point of attack in attempting to uncover the mechanism of the physiological action of vitamin A seemed to lie in an analysis of the phospholipids, or more specifically in an analysis of the amount and degree of unsaturation of the phospholipid fatty acids in the tissues of normal animals as compared with vitamin A-deficient animals.

On the assumption that the phospholipid content of tissues is constant and characteristic in amount for any one type of tissue, independent of the nutritional state of the animal, any marked change from the normal condition in the phospholipid content of vitamin A-deficient animals must be ascribed to the specific action of this vitamin. This assumption would appear to be justified by a large amount of experimental work. Rubow (1904) found that the lecithin content of heart and kidney was maintained constant in amount after fasting, and after phosphorus and chloroform poisoning. Shioji (1924) found that changing the type of fat in the diet had no effect on the amount, and very little effect on the composition of the phospholipid fatty acids. Morita (1924) demonstrated that there was no change in the amount of phospholipids present in the tissues as a result of lecithin feeding. The French workers (Mayer and Schaeffer, 1913; Terroine and Belin, 1927; Terroine, Hatterer, and Roehrig, 1930; Terroine and Hatterer, 1930) have repeatedly emphasized the existence of a constant fatty element, identified with the phospholipid fraction, in the tissues of animals fasted until death. Sinclair (1930, 1932) found that the composition of phospholipids in animal tissues was influenced to a slight extent by the character of fat in the diet, but that the amount of phospholipids was unaffected, except in the case of the liver, where a slight decrease from the normal was obtained in rats on fat-free diets.

Actually, it was found in this investigation that removal of vitamin A from the diet did indeed cause a considerable decrease in the phospholipid fatty acid content of the tissues analyzed. However, contrary to expectations, the data show that there was no oxidative destruction of the fatty acids either of the fat stores of the body, or of the neutral fats of the liver, which are presumably immediately available for synthesis into phospholipids, or of the formed phospholipids themselves in any of the tissues analyzed. Therefore, despite the many references in the literature attesting to the constant character of the phospholipids under various dietary conditions, it was nevertheless considered necessary to include in this investigation a study of other types of dietary deficiencies (lack of vitamins B<sub>1</sub>, B<sub>2</sub>, and D, and acute fasting) in order to determine whether the observed decrease was due specifically to the lack of vitamin A, or whether, on the other hand, it might not be a more general phenomenon accompanying stunted growth and other abnormalities.

#### EXPERIMENTAL

##### *Procedure*

*Care and Condition of Experimental Animals*—Albino rats were used as experimental animals since methods of inducing the various types of vitamin deficiencies are well standardized for this species. The normal controls, fasted, and vitamin A-free rats were raised in this laboratory. The basal diet had the following composition.

	gm.
Rice starch. ....	500
Purified casein (Osborne and Mendel, 1920-21). ....	200
Linseed oil. ....	200
Salt mixture (Hume and Smith, 1928). ....	50

The control animals received 500 mg. of dried yeast (Northwestern Yeast Company) and 4 to 5 drops of Patch's cod liver oil each day. 1 drop of a commercial preparation of viosterol was substituted for the cod liver oil in the diet of the group to be deprived of vitamin A.

The animals to be fasted were taken from the normal control group. It was found that the abrupt removal of all food from such normal rats caused death generally within 3 days, before any noticeable depletion of the fat stores had occurred. If, on the



other hand, the amount of food was reduced gradually, the life of the rat might be prolonged until the macroscopically visible fat stores in the peritoneal cavity were partially or completely depleted. Thus, in a series of five rats, the period of fasting (or of greatly reduced food intake) varied from 3 days to 2 weeks, and the weight lost during fasting ranged from 6 to 45 per cent of the original weight. Nevertheless, it may be said that all of these animals were fasted to the point of death, since all were extremely weak at the time they were killed.

The rats deficient in vitamins B<sub>1</sub>, B<sub>2</sub>, and D were obtained from outside sources.<sup>1</sup> The vitamin B-free diet had the following composition.

	<i>per cent</i>
Dextrin.....	62
Purified casein.....	18
Crisco.....	14
Salt mixture (Osborne and Mendel, 1919).....	4

The vitamin B<sub>1</sub> concentrate (Bourquin and Sherman, 1931) was supplied to those rats which were to be deprived only of vitamin B<sub>2</sub>, and the vitamin B<sub>2</sub> concentrate (autoclaved yeast) was supplied to the group to be deprived of vitamin B<sub>1</sub>. All rats were in advanced stages of avitaminosis at the time they were killed for analysis.

The vitamin D-free group was maintained on Steenbock's rachitogenic diet, Ration 2965 (Steenbock and Black, 1925).

	<i>per cent</i>
Ground yellow corn.....	76
Wheat gluten.....	20
CaCO <sub>3</sub> .....	3
NaCl.....	1

At the end of 22 days, Roentgenograms were made of each rat to determine the degree of calcification. Those animals showing marked symptoms of rickets were killed for analysis of tissue phospholipids. Except for their rachitic condition, these rats were entirely healthy in appearance and normal in weight.

<sup>1</sup> These animals, and the records concerning their care and condition quoted above, were obtained from the Biological Laboratories of the Anheuser-Busch Company of St. Louis, through the courtesy of Mr. Carrol Neff.

*Selection and Preservation of Tissues for Analysis*—Only tissues containing a relatively high percentage of phospholipid (liver, kidney, lung, and spleen) were selected for analysis. Brain was not used because of the difficulty of separating the large cerebroside fraction from the phospholipids. Since 2 to 5 gm. of moist tissue were required for an accurate analysis of the phospholipid fatty acids, the tissues of two or more rats were often combined for a single analysis. The rats were asphyxiated and bled to death. The tissues were dissected out, rinsed in Ringer's solution, weighed as rapidly as possible, and immediately placed in thermos containers provided with solid carbon dioxide, where they were preserved for analysis. It is believed that this method of preservation precluded the possibility of enzymatic breakdown of the phospholipids and oxidation of the constituent fatty acids during the storage period. It is also probable that this treatment facilitated the subsequent extraction process by mechanical disruption of the cells caused by sudden freezing and thawing.

*Method of Analysis*—The procedure followed in obtaining the phospholipid fatty acids was similar to that described by Sinclair (1929). The tissues were first exhaustively extracted with hot alcohol in a continuous extraction apparatus. The alcohol was then removed by evaporation, and the residue, which presumably contained all the fatty substances originally present in the tissues, was taken up in ethyl ether. The phospholipids were precipitated by the addition of acetone and  $MgCl_2$ , and separated from the acetone-soluble portion (neutral fat and cholesterol) by centrifuging. In the case of the liver both fractions were saved and saponified separately; in the case of the other tissues only the acetone-insoluble fraction was saponified, the neutral fats being discarded. The fatty acids were freed from the soap solution by acidification and subsequent extraction with petroleum ether. After removal of the petroleum ether by distillation, the fatty acids were dried *in vacuo* and weighed. These fatty acids, obtained from the saponification of all the insoluble material, will henceforth be designated phospholipid fatty acids.

Iodine numbers were determined by the pyridine sulfate dibromide method originally described by Rosenmund and Kuhn-henn and quoted by Yasuda (1931-32). Several determinations were made of the degree of unsaturation of neutral fat from the

intraperitoneal fat stores of normal and vitamin A-deficient rats in order to determine whether any oxidative destruction of the more highly unsaturated fatty acids might have occurred in the absence of the vitamin. The fat was saponified directly and the fatty acids freed and extracted as described above.

### Results

The iodine number of the fatty acids obtained from the intraperitoneal fat of both normal and vitamin A-deficient rats (Table I) is higher than that generally obtained from the body fat of rats on a stock diet. This is to be expected, since the experimental diets in this case contained 20 per cent linseed oil (iodine number about 190). Anderson and Mendel (1928) have shown that the

TABLE I  
*Iodine Number of Fatty Acids from Intraperitoneal Fat of Normal and Vitamin A-Deficient Rats*

Normal controls	Vitamin A-deficient animals
131	135
143	139
146	144
139	153
144	140
Average 140	142

quality of fat produced in the animal body tends to resemble that in the diet. However, the point of main interest in these data is that the fat of the vitamin-deficient rats is quite as unsaturated as that of the normal controls.

The effect of vitamin deficiencies on the fatty acids of the liver is demonstrated in Table II. A marked decrease is apparent in the phospholipid fatty acid content of the liver in all types of vitamin deficiencies except vitamin D.

The non-phospholipid liver fatty acids vary considerably in amount even in the normal control rats, but in general the amount is much greater in the vitamin-deficient rats than in the controls. In the case of two vitamin A-deficient rats, both of which were very weak, although not especially emaciated, the neutral fat

TAB.  
*Effect of Vitamin Deficiencies on Fatty Acids of Liver*

Dietary condition	No. of rats analyzed	No. of separate analyses	Phospholipid fatty acids			Non-phospholipid fatty acids		
			Per gm. wet tissue	Per gm. dry extracted tissue	Iodine No.	Per gm. wet tissue	Per gm. dry extracted tissue	Iodine No.
Normal control.....	7	6	mg. 18 (16-19.9)	mg. 92.8 (82-97)	145 (135-155)	mg. 26.7 (21-37)	mg. 136.6 (114-190)	139 (122-150)
Rachitic.....	2	1	16.7	93.3	139	60.4	336.8	138
Vitamin A-deficient....	9	7	11.5 (9.5-14.6)	61.9 (55-78)	146 (140-155)	69.9 (32-143)	366.9 (165-871)	164 (154-176)
Vitamin B <sub>1</sub> -deficient....	4	1	15.7	84.4	147	70.7	378.6	178
Vitamin B <sub>2</sub> -deficient....	3	1	7.5	43.6	168	51.2	296.7	156
Deficient in vitamin B complex.....	4	1	6.9	36.8	150	23.4	123.5	141

content amounted to almost 50 per cent of the dry weight of the liver. Such fatty infiltrations of the liver have frequently been described in pathological conditions involving abnormal fat metabolism (Hartley and Mavrogordato, 1908; Sebrell, 1929).

In comparing the iodine numbers, it must be pointed out that the fat content of the vitamin-deficient diets differed in most cases from that of the normal diet. Despite the very great differences in the amount and kind of fat fed, it is evident that the iodine number of the phospholipid fatty acids is remarkably constant throughout, indicating no oxidative destruction of the unsaturated fatty acids in the vitamin A-deficient group.

TABLE III  
*Effect of Fasting on Fatty Acids of Liver*

Days of fasting	Per cent of original weight lost during fasting	Phospholipid fatty acids			Non-phospholipid fatty acids		
		Per gm. wet tissue	Per gm. dry extracted tissue	Iodine No.	Per gm. wet tissue	Per gm. dry extracted tissue	Iodine No.
		mg.	mg.		mg.	mg.	
3	6.4	17.8	91.4	135	50.7	260.1	144
6	14.1	17.4	82.8	138	40.6	194.0	148
7	17.9	14.9	74.3	132	28.1	140.2	142
8	28.7	17.1	79.1	145	25.3	117.0	153
13	44.3	10.1	53.8	160	83.9	445.9	170

Table III contains the results of similar analyses of the livers of fasted rats. It is to be remembered that all of these rats were fasted to the point of death, even though the number of days of fasting and the weight lost during fasting varied over wide intervals. In considering the phospholipid fatty acid content of the livers, it is apparent that a rat which had lost only 6 per cent of its weight showed no decrease from the normal in the phospholipid fatty acid content of the liver. However, as the animals were made to lose greater and greater percentages of their weight through more gradual fasting, the phospholipid fatty acid content of the liver steadily decreased.

The neutral fat, on the other hand, showed the same wide variations that were found in the vitamin-deficient animals. It is especially interesting that the rat which had lost the most weight

and was extremely emaciated, nevertheless contained more than three times the normal amount of neutral fat in the liver.

That the reduction in phospholipid content caused by dietary deficiencies is not confined to the liver is shown in Table IV. Kidney, lung, and spleen show similar reductions in the phospho-

TABLE IV

*Effect of Dietary Deficiencies on Phospholipid Fatty Acids of Kidney, Lung, and Spleen*

Organ	Dietary condition	No. of rats analyzed	No. of separate analyses	Phospholipid fatty acids		
				Per gm. wet tissue	Per gm. dry extracted tissue	Iodine No.
Kidney	Normal control	8	3	mg. 15.5 (15.1-15.7)	mg. 92.9 (89-99)	113 (107-120)
	Fasted	3	1	14.2	87.2	101
	Rachitic	5	1	14.9	95.6	114
	Vitamin A-deficient	8	2	10.2 (9.8-10.7)	67.1 (61-73)	107 (104-111)
	Deficient in vitamin B complex	4	1	8.7	50.1	124
Lung	Normal control	10	3	13.9 (13.3-15)	92.7 (83-102)	92 (83-101)
	Fasted	5	1	14.2	86.3	84
	Rachitic	5	1	12.8	83.1	83
	Vitamin A-deficient	18	3	12.0 (10.9-13.1)	76.9 (67-86)	88 (80-93)
	Deficient in vitamin B complex	10	1	11.1	71.3	109
Spleen	Normal control	12	2	9.4 (9.3-9.4)	53.5 (53-56)	116 (113-118)
	Vitamin A-deficient	19	2	7.1 (5.7-8.5)	39.9 (31-48)	121 (115-127)

lipid fatty acids whenever the diet is such as to interfere with the growth of the animal, although the decrease is not so marked as in the case of the liver. The values obtained from kidney and lung of fasted rats are not so significant in this case, since the tissues from several rats which had lost varying percentages of weight had to

be combined for the analyses. As in the case of the liver, no significant difference was obtained in the iodine numbers of the phospholipid fatty acids of control and experimental animals.

#### DISCUSSION

The results demonstrate no specific connection between vitamin A and phospholipid metabolism. The observed decrease in the phospholipid content of the tissues of vitamin-deficient rats was not specifically connected with the absence of this vitamin, and was not accompanied in the vitamin A-deficient rats by the decrease in the iodine number which might have been expected on the basis of the observed *in vitro* action of this vitamin. No adequate interpretation can yet be given of the results of the *in vitro* experiments, which indicated that vitamin A acts as an antioxidant towards unsaturated fatty acids.

It is clearly demonstrated, however, that the phospholipid content of tissues is not independent of diet, but that, on the contrary, it may be decreased by a number of diets inadequate for normal growth. In fact, there appears to be a certain amount of correlation between loss in weight, or lack of normal gain in weight, and decrease in the phospholipid fatty acid content of the tissues. Fig. 1 illustrates this point in the case of the liver. There appears to be a constant value for the phospholipid fatty acids from the livers of rats which are growing normally on an adequate diet; but as soon as the growth rate declines, irrespective of the type of dietary deficiency responsible for the decline, the phospholipid fatty acid content of the liver also decreases.

This point has a distinct bearing on current views concerning the function of tissue phospholipids. If it were true, as has generally been supposed, that the phospholipids of the tissues are very constant in amount, and disappear during fasting only at the same rate as the proteins, then it would be reasonable to conclude that the phospholipids are so built into the structure of the protoplasm that it is impossible to decrease their amount without causing the disintegration and subsequent death of the cell. This is the view developed by the French physiologists Mayer and Schaeffer, and Terroine and his coworkers, as a result of their experimental work which pointed to the existence in fasted animals of an irreducible minimum of fatty material. This material, called the *constant*

element, was identified with the phospholipid fraction from considerations of the fatty acid to phosphorus ratio, and of the iodine number.

The results of the present investigation are not in accord with these interpretations. Since it has been found possible to reduce the phospholipid fatty acid content of tissues to a value 60 per cent below normal, it seems hardly conceivable that all of the

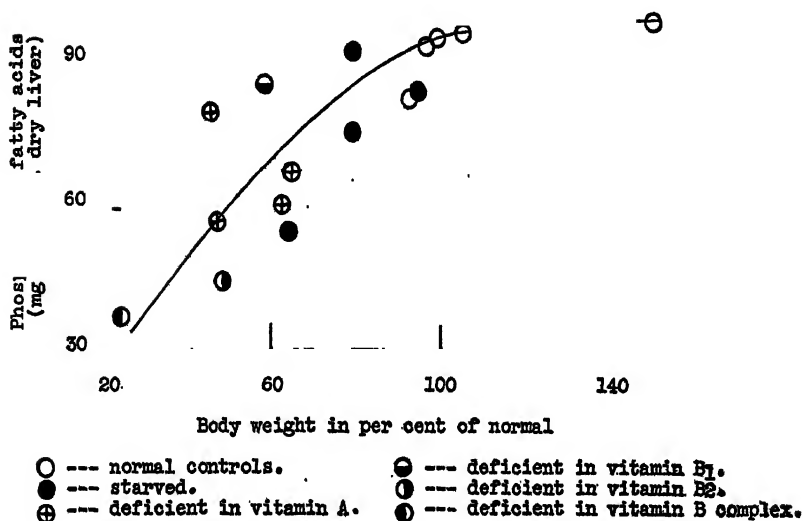


FIG. 1. The effect of subnormal growth on the phospholipid fatty acids of the liver. The values plotted on the abscissa were obtained by dividing the body weight of the experimental rats at the time they were killed by the normal body weight of rats of the same age, obtained from tables compiled by Donaldson (1924).

phospholipids originally present could actually have been built into the architecture of the cell protoplasm in a form indispensable for the functioning of the cell.

An entirely different view of the function of the phospholipids was first suggested by Loew (1891); namely, that tissue phospholipids act as intermediate products in fat metabolism, serving as a conveying mechanism for the fatty acids destined for subsequent oxidation in the tissues. The evidence for this theory is mainly



inferential: the greater miscibility with water and absorbability of the lipids as compared with the fats, and the comparative ease with which they are hydrolyzed to their component parts by acid and by lipases. In addition, Bloor (1916) has shown that blood lecithin rises during fat absorption, and Meigs, Blatherwick, and Cary (1919) found that during milk fat secretion in cows the difference in the phospholipid content of the blood passing to and from the mammary gland was sufficient to account for all of the fat secreted in the milk.

Such a view does not require the maintenance of a constant amount of phospholipid in each tissue. Under normal conditions, fat is probably supplied to an actively functioning organ at the same rate that it is utilized for the liberation of energy; hence, the phospholipid content of the organ would be fairly constant. But any condition causing a disturbance in fat metabolism might very well upset this equilibrium.

Obviously, the mechanism of fat utilization was not functioning normally in the vitamin-deficient rats analyzed in this investigation. The extreme emaciation of the animals and the marked fatty infiltrations of the livers in many cases evidenced serious disturbances in fat metabolism. It is not surprising, therefore, if it be assumed that the phospholipids are intermediate products in fat metabolism, that their amount should have been greatly decreased in the tissues of the vitamin-deficient rats. However, in the absence of further evidence on the nature of the disturbance in fat metabolism, it is hardly profitable to speculate on the specific cause of the decrease in phospholipids.

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#### SUMMARY

Gravimetric analyses demonstrated that, whereas there is a constant value for the phospholipid fatty acids in a given tissue of rats which are growing normally on an adequate diet, as soon as the growth rate falls off, irrespective of the type of dietary deficiency responsible for the falling off, the phospholipid fatty acid content

of the tissues also decreases. Iodine number determinations indicated no difference in the degree of unsaturation of the fatty acids of the vitamin-deficient as compared with the normal tissues.

The relation of these data to current views on the function of phospholipids is discussed. It is pointed out that the results do not support the theory that the phospholipid content of cells is irreducible in amount and represents an indispensable part of the protoplasmic structure. On the other hand, the results are entirely consistent with the view that the phospholipids act, for the most part, as intermediate products in fat metabolism.

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## A COMPARISON OF THE WU AND KJELDAHL METHODS OF SERUM PROTEIN DETERMINATION

BY L. R. TUCHMAN AND HARRY SOBOTKA

*(From the Laboratories of the Mount Sinai Hospital, New York)*

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A colorimetric method for the determination of the albumin, globulin, and fibrinogen in plasma and serum has been based by Wu (1922; cf. Wu and Ling, 1927, 1928) on the direct estimation of tyrosine in proteins by means of the "phenol reagent." The value of the method depends on the constancy of the tyrosine content of the protein fractions, and Wu accordingly determined the average "tyrosine factors" for the albumin, globulin, and fibrinogen of human blood and the blood of a number of animal species.

When the Wu method was applied in this laboratory to determinations on blood specimens from the clinical services of the hospital, specimens showing low total protein values occasionally failed to display the reduction in the albumin to globulin ratio 1:1 and below, found with the customary Kjeldahl determination (1883). This discrepancy between the two methods could hardly have been due to errors of procedure, but pointed towards changes in the composition of the protein fractions under pathological conditions. We had earlier investigated whether such qualitative changes, especially in edematous conditions, could be detected without resorting to previous hydrolysis of the proteins. No conclusive results could be reached by the Willstätter-Waldschmidt-Leitz titration method in alcohol for free carboxyl groups (1921) and by Linderstrøm-Lang's titration in acetone (1928) and Van Slyke's method (1911) for free amino groups of diamino acids. As the apparent discrepancies between the values found by the Wu and Kjeldahl methods may be related to such a qualitative change in the proteins, a parallel series of determinations in normal and abnormal cases should throw light on the complex question of protein structure in edematous states and

simultaneously demonstrate how far the Wu method can be relied upon.<sup>1</sup>

### *Method*

Duplicate or triplicate determinations were carried out on blood serum, no values being recorded unless they checked within 2 per cent. These determinations were carried out on filtrates obtained from single cc. samples of serum, so that total protein and albumin to globulin ratio by both methods required only 2 cc. of serum. The procedure used for albumin in serum follows.

To 1 cc. of serum are added 9 cc. of a saturated magnesium-sodium sulfate solution.<sup>2</sup> The solutions are mixed and allowed to stand for an hour or more in a warm place and then filtered until clear, leaving only albumin in solution. 1 cc. of the filtrate is diluted with 10 cc. of water, 1 cc. of a 10 per cent solution of sodium tungstate and 1 cc. of  $\frac{2}{3}$  N sulfuric acid are added, and the solution is centrifuged after 10 minutes of standing. The precipitated albumin is redissolved by the aid of 1 drop of saturated sodium carbonate solution in 10 cc. of water. This solution is transferred to a volumetric flask of 25 cc. capacity and 0.5 cc. of phenol reagent (Wu, 1922) is added, followed by 1 cc. of 10 per cent sodium hydroxide. Water is added to the 25 cc. mark, the solution is thoroughly mixed, and after 10 minutes compared in a colorimeter against a standard tyrosine solution prepared as follows: 1 cc. of a 0.02 per cent tyrosine solution plus 0.5 cc. of phenol reagent with 20 cc. of water and 1 cc. of solution of 10 per cent sodium hydroxide were made up to 25 cc.

For the determination of total protein, 1 cc. of serum is diluted to 15 cc. with water; 1 cc. of the resulting solution is treated as above with tungstate and sulfuric acid, the precipitate redissolved

<sup>1</sup> J. P. Peters and D. D. Van Slyke (Quantitative clinical chemistry, Baltimore, 2, 693 (1932)) report that one of their colleagues has experienced similar deviations with the Wu method.

<sup>2</sup> This double salt was first suggested by Halliburton (1884-85) for protein fractionation. Unpublished studies by Sobotka and Reiner (*cf.* Reiner and Shwartzman, 1929), showing the interchangeability of the saturated solutions of  $MgNa_2(SO_4)_2$  and of  $(NH_4)_2SO_4$  in the fractionation of plasma proteins, justify the use of this  $NH_3$ -free sulfate which greatly simplifies the Kjeldahl determination without certain disadvantages inherent in other sulfates.

and the blue color elicited with phenol reagent and sodium hydroxide is read against the tyrosine standard. The micro-Kjeldahl determinations were carried out on 1 cc. of the albumin filtrate, or on 1 cc. of the 1:15 diluted serum. The non-protein nitrogen was separately estimated and subtracted, but this correction was always very small.

### *Results*

Twenty-five serum specimens were analyzed from eighteen cases, consisting of a group with normal total proteins and a group with low total proteins with or without visible edema, including a case of nephrosis proved at autopsy. A case of kala-azar was studied because this disease is supposed to show high total protein figures with a high globulin percentage.

Figures are presented in Tables I and II. The group with total protein values of 6 per cent or over, which we have arbitrarily taken as the lower limit of normal blood protein, includes cases with kidney damage, hypertension, pernicious anemia, and also a nephrosis in the interval stage. The tyrosine factors for albumin and globulin in this group were 21.0 and 16.2, respectively, in satisfactory agreement with 21.4 and 15.8, the corresponding factors given by Wu. Except for Case 10 (nephrosis in interval) and for the globulin factor in Case 9 (kala-azar), the deviations are small and fall within the margin of experimental variation of the method.

A different situation confronts us in Cases 11 to 18 (Table II). Here, considerable deviations are encountered when the Kjeldahl values are compared with the figures calculated from the tyrosine content on the basis of Wu's factors. This group includes a number of cases of nephritis with a definite nephrotic picture and also two non-renal cases, a case of pernicious anemia with ascites, and a very marked gastrointestinal intoxication in an infant. Case 18 represents three determinations at intervals on the proved case of nephrosis.

The second to seventh columns of Table I and the third to eighth columns of Table II contain the values for total protein, albumin, and globulin. (While we assume the Wu values to be incorrect in the edematous cases in Table II, they are given to illustrate the extent of the error.) The albumin percentage appears some-

times as much as 0.7 per cent too high, the globulin percentage maximally by the same amount too low with the Wu method. In regard to total proteins, both errors compensate each other in a certain measure. Thus, the total proteins as determined colorimetrically, do not deviate more than 10 per cent from the Kjeldahl

TABLE I

*Comparison of Wu and Kjeldahl Methods in Cases with Normal Serum Protein Percentage*

Case No.	Total protein		Albumin		Globulin		Albumin:glob- ulin ratio		Tyrosine factors	
	Kjel- dahl	Wu	Kjel- dahl	Wu	Kjel- dahl	Wu	Kjel- dahl	Wu	Albu- min	Glob- ulin
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	7.4	7.4	4.2	4.1	3.2	3.3	1.31	1.24	21.9	15.3
2	6.8	7.3	3.6	3.6	3.2	3.7	1.12	0.97	21.4	13.7
3	6.8	6.2	4.0	4.0	2.8	2.2	1.43	1.82	21.4	20.1
4	6.7	6.8	4.3	4.5	2.4	2.3	1.79	1.95	20.4	16.5
5	6.6	6.5	4.5	4.5	2.1	2.0	2.14	2.25	21.4	16.2
6	6.2	6.7	3.7	4.1	2.5	2.6	1.48	1.58	19.3	15.2
7	6.2	6.3	3.8	3.9	2.4	2.4	1.58	1.62	20.9	15.8
8	6.2	6.1	3.8	3.8	2.4	2.3	1.58	1.65	21.4	16.5
Average.....									21.0	16.2
Wu's factors.....									21.4	15.8
9, kala-azar	7.3	6.4	3.2	3.2	4.1	3.2	0.78	(1.00)	21.4	<b>20.5</b>
10, nephrosis, interval stage	7.3	7.1	3.8	4.5	3.5	2.6	1.08	(1.73)	<b>18.1</b>	<b>21.3</b>
	7.6	7.1	5.1	4.7	2.5	2.4	2.04	(1.96)	23.2	16.5

The misleading albumin to globulin quotients are given in parentheses. Inversion of tyrosine factors of albumin and globulin is indicated by bold-faced figures.

values except in the first determination on Case 16. However, the albumin to globulin quotient is misrepresented in most cases of Table II by Wu's method (these quotients are given in parentheses). The last two columns in each table give the factors computed for each individual specimen. The albumin factors are lower throughout Table II, reaching values as low as 15.6 and 15.4,

indicating an increase in tyrosine content from 4.7 per cent to above 6 per cent. Contrariwise, the tyrosine factors for globulin rise above the normal value of about 16 to 21 and even 23. In six

TABLE II

*Protein Partition by Kjeldahl Method and by Wu Method in Edematous Cases*

Case No.	Date	Total protein		Albumin		Globulin		Albumin: globulin ratio		Tyrosine factors	
		Kjeldahl	Wu	Kjeldahl	Wu	Kjeldahl	Wu	Kjeldahl	Wu	Albumin	Globulin
	1931	per cent	per cent	per cent	per cent	per cent	per cent				
11, nephrotic stage of chronic nephritis	July 25	4.0	4.1	1.7	2.1	2.3	2.0	0.74	(1.05)	17.3	18.2
	" 29	4.5	4.6	1.9	2.6	2.6	2.0	0.73	(1.30)	15.6	20.5
	Aug. 3	4.6	4.6	1.8	2.5	2.8	2.1	0.64	(1.19)	15.4	21.1
	" 18	3.8	4.1	2.3	2.8	1.5	1.5	1.55	(1.86)	18.9	15.8
12, gastrointestinal intoxication	" 19	4.4	4.5	2.5	3.2	1.9	1.3	1.31	(2.46)	16.9	23.1
13, nephrotic stage of nephritis	" 7	5.6	5.6	3.1	3.5	2.5	2.1	1.25	(1.67)	19.0	18.8
14, pernicious anemia, ascites	" 18	5.6	5.5	3.6	3.9	2.0	1.6	1.80	(2.44)	19.7	19.3
15, edema	" 19	4.6	4.3	2.5	2.6	2.1	1.7	1.19	(1.53)	20.6	19.4
16, nephrotic stage of nephritis	" 9	4.4	5.0	1.8	2.1	2.6	2.9	0.68	(0.72)	18.4	14.1
	Sept. 25	4.5	4.3	2.1	2.2	2.4	2.1	0.88	(1.05)	20.4	18.1
17, chronic nephritis	" 18	4.2	4.3	1.8	2.0	2.4	2.3	0.75	(0.84)	19.3	16.5
	" 25	4.4	4.3	1.4	1.4	3.0	2.9	0.47	(0.48)	21.4	16.4
	1933										
18, nephrosis	Feb. 8	4.4	4.7	1.5	2.0	2.9	2.7	0.52	(0.74)	16.0	17.0
	" 20	4.3	4.6	1.5	1.9	2.8	2.7	0.53	(0.70)	16.9	16.4
	" 25	4.3	4.6	1.7	2.1	2.6	2.5	0.65	(0.84)	17.3	16.4
Average.....										(18.2)	(18.1)

The misleading albumin to globulin quotients are given in parentheses. Inversion of tyrosine factors of albumin and globulin is indicated by bold-faced figures.

specimens from four cases the values pass each other. The averages are 18.2 for albumin and 18.1 for globulin. These average figures have illustrative value only, and in face of the wide individual variations may not be used as "corrected factors."



It may be noted that in the case of genuine nephrosis the albumin sank markedly while the globulin factor was little changed from normal. The case of kala-azar (Table I, Case 9) showed lower globulin and lower total protein values by the Wu method than by the micro-Kjeldahl method with a normal albumin factor of 21.4 and a globulin factor calculated at 20.5, as compared with Wu's 15.8.

#### DISCUSSION

In examining these results one is inclined to pronounce the Wu method satisfactory in cases with total serum protein in excess of 6 per cent. In cases with low blood protein, especially in obviously edematous conditions, its use for the determination of the albumin to globulin ratio should be avoided.

The reason for its analytical failure in these instances must be deeply rooted in the mechanism of edema. The albumin seems enriched in tyrosine while the globulin fractions as a total lose part of their comparatively high tyrosine content.<sup>3</sup> Whether these shifts in "tyrosine" content are brought about by actual destruction and synthesis of protein molecules or whether they are the result of shifts among the several fractions of globulin and of albumin respectively, will be investigated by tyrosine analysis of such subfractions in normal and diseased serum. The theoretical significance of our results will be treated in detail elsewhere (Sobotka, 1932). If the above interpretation is correct, these results provide the first experimental evidence for qualitative changes of blood protein in addition to the known quantitative changes in edema. It throws no light, however, on whether the loss of albumin through the kidneys is the primary factor in the etiology of nephrosis, or whether as A. A. Epstein (*cf.* Leiter, 1931) suggests, changes in the systemic protein metabolism are the underlying causes.

#### CONCLUSIONS

1. The Wu method for determining total protein and albumin to globulin ratio of the serum fractions by their tyrosine content

<sup>3</sup> It should be remarked that the color reaction with the phenol reagent is not absolutely specific for tyrosine and that other amino acids, particularly tryptophane, are responsible for a minor share of the blue color.

is accurate for clinical purposes in cases with serum protein of 6 per cent or higher.

2. In cases of nephrosis or nephritis with edema, the Wu method gives divergent results and its sole use in these cases is dangerous.

3. The deviations can be explained by pathological changes in the chemical composition of the serum proteins.

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# THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

## XXXI. THE COMPOSITION OF THE ACETONE-SOLUBLE FAT OF THE TIMOTHY BACILLUS\*

BY MARY C. PANGBORN,† ERWIN CHARGAFF,‡ AND  
R. J. ANDERSON

*(From the Department of Chemistry, Yale University, New Haven)*

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### INTRODUCTION

Detailed analyses of the fat or lipid fractions of the non-pathogenic acid-fast timothy bacillus have never been published. These compounds are, however, of practical and scientific interest in connection with the comparative chemical and biological investigations on tubercle bacilli which are being conducted in this laboratory and in various other research institutions under the auspices of the Medical Research Committee of the National Tuberculosis Association.

The separation of the lipid fractions of the timothy bacillus into phosphatide, acetone-soluble fat, and wax has been reported in a former paper (1) and the composition of the phosphatide has already been determined (2). The present report deals with the composition of the fraction designated acetone-soluble fat.

The fat was saponified and the cleavage products were sep-

\* An abstract of this paper was read at the meeting of the American Society of Biological Chemists at Montreal, April, 1931.

The data are taken from the dissertation submitted by Mary C. Pangborn to the Faculty of the Graduate School, Yale University, 1931, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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‡ Holder of the Milton Campbell Research Fellowship at Yale University, 1929-30.

arated into a water-soluble fraction, unsaponifiable matter, and fatty acids.

The fat was evidently not a glyceride because we were unable to identify any glycerol in the water-soluble fraction. Some substance other than glycerol was present, possibly a carbohydrate or a polyhydric alcohol, but the substance could not be identified. The water-soluble constituents contained a trace of volatile fatty acids, but the amount was so small that it was impossible to identify any definite acid.

The unsaponifiable matter was a dark colored oil with an iodine number of 126. It did not solidify on cooling, and gave none of the usual sterol color reactions.

The fatty acids consisted of solid saturated acids and liquid acids. The solid saturated fatty acids consisted principally of palmitic acid. A small quantity of a higher acid, possibly stearic acid, was also present, but the amount was too small for a definite identification. A higher optically active hydroxy acid was isolated, but could not be identified with any known acid of this type.

The liquid fatty acids had an iodine number of 65 and contained, therefore, unsaturated acids, but the principal portion of the liquid acids was a liquid saturated fatty acid which appeared to be identical with tuberculostearic acid (3). The separation of the unsaturated fatty acids from the liquid saturated acid presented in this case unusual difficulties because it was found to be impossible to effect the catalytic reduction of the crude unsaturated acids because some substance was evidently present which poisoned the catalyst. In order to secure a smooth and rapid reduction of the unsaturated acids, it was necessary to convert the mixed liquid acids into methyl esters and distil the esters in a high vacuum. After complete reduction had been effected, the reduced acids were separated by the lead soap-ether method.

While we have no direct evidence regarding the number of double bonds in the unsaturated fatty acids, it is possible, nevertheless, from the data at hand to draw some conclusions. The crude liquid acids weighed 37.6 gm. and the iodine number was 65. If the unsaturated fatty acid consisted only of a  $C_{18}$  acid with one double bond, the mixture should, on catalytic reduction, yield 24.4 gm. of palmitic acid, while, if two double bonds were present, the amount of palmitic acid should be 12.2 gm. In case oleic

acid had been the only unsaturated acid present in the mixture, there should have been formed 27.1 gm. of stearic acid on reduction. It is evident, therefore, since the total amount of the reduced acids weighed only 9.9 gm., that very little, if any, unsaturated acids with one double bond could have been present. The greater portion of the unsaturated acids must have contained two or three double bonds.

The reduced fatty acid consisted principally of palmitic acid. A small quantity of a higher acid was present, but it could not be identified and no pure stearic acid could be isolated.

The liquid saturated fatty acid which was isolated after removing the reduced acid was purified by repeated fractionation of its methyl ester. The purified acid corresponded in composition to a saturated fatty acid of the formula  $C_{18}H_{36}O_2$ , and we believe that it is identical with tuberculostearic acid (3). No optically active acid similar to phthioic acid could be found in the liquid acid fraction. The fat from the timothy bacillus in this respect is similar to the fat from the bovine tubercle bacillus (4).

The fatty acids which have been isolated and identified in the fat from the timothy bacillus consist, therefore, principally of palmitic acid, an unsaturated  $C_{16}$  acid which yields palmitic acid on reduction, and a saturated liquid acid which is isomeric with stearic acid. Very small amounts of higher acids are present, but they could not be identified, and finally a higher optically active hydroxy acid is present in the solid saturated fatty acid fraction.

#### EXPERIMENTAL

The acetone-soluble fat was a dark reddish brown oil which solidified when cooled to  $-10^{\circ}$ . It contained a trace of phosphorus, but was free from sulfur and nitrogen. The following constants were determined, by use of the methods described in the Manual of the Association of Official Agricultural Chemists (5).

##### *Constants of Acetone-Soluble Fat*

Iodine No.....	72.3
Acid No.....	59.4
Saponification No.....	157.5
Reichert-Meissl ".....	1.98
Unsaponifiable matter, per cent.....	27.0

It was difficult to determine accurately the acid number and the saponification number owing to the dark color of the solutions. The titrations were made, alkali blue being used as indicator. That the amount of free fatty acids is high was corroborated by the results obtained on extracting an ethereal solution of the fat with dilute sodium hydroxide. The fatty acids isolated from the alkaline extract were equivalent to about 30 per cent of the fat.

### *Saponification of Fat<sup>1</sup>*

The fat was saponified by refluxing with alcoholic potassium hydroxide in an atmosphere of nitrogen. The cleavage products were isolated in the manner described in Paper XXIV (4). The separation of the fatty acids into solid saturated acids and liquid

TABLE I  
*Composition of Acetone-Soluble Fat from Timothy Bacillus*

	Lot I 41.6 gm. of fat		Lot II 41 gm. of fat	
	gm.	per cent	gm.	per cent
Unaponifiable matter.....	9.2	22.1	11.1	27.0
Fatty acids.....	30.8	74.0	28.1	68.5
Water-soluble constituents.....	2.0	4.8	1.3	3.1
Solid saturated fatty acids.....	6.6	15.9	9.4	22.9
Liquid fatty acids.....	19.4	46.6	18.2	44.4
Reduced " " .....	5.0	12.0	4.9	11.9
Liquid saturated fatty acids.....	8.0	19.2	9.6	23.5

acids was accomplished by means of the lead soap-ether method. The liquid fatty acids were converted into the methyl esters and the latter, after they had been distilled, were reduced with hydrogen and platinum oxide (6). The ester was then saponified and the acids separated into solid reduced acids and liquid saturated acids by repeating the lead soap-ether treatment. The various operations are described in more detail in the paper referred to above.

Two lots of fat weighing 41.6 gm. and 41 gm. respectively were saponified and the results obtained are given in Table I.

<sup>1</sup> During all operations until the liquid acids were reduced, the fatty acids were protected from air by an atmosphere of carbon dioxide or nitrogen. All solvents had been freshly distilled and the alcohol had been distilled over potassium hydroxide.

*Volatile Fatty Acids*

The aqueous solution which remained after the fatty acids had been extracted with ether was concentrated by distillation and examined for water-soluble constituents, as will be described later. The distillate was made slightly alkaline with potassium hydroxide and concentrated to a volume of about 100 cc. The solution was acidified with sulfuric acid and distilled over a free flame until the distillate was neutral. The distillate was neutralized with potassium hydroxide and evaporated to dryness, when a residue was obtained which weighed 0.26 gm. When some of this substance was treated with sulfuric acid, the odor of acetic acid could be detected and there was also a slight suggestion of the odor of butyric acid. The fat evidently contains traces of volatile water-soluble acids, but the amount was very small and no definite acids could be identified.

*Water-Soluble Constituents*

The acid aqueous solution mentioned in the preceding paragraph was neutralized with potassium hydroxide, concentrated under reduced pressure to dryness, and the residue freed from moisture as far as possible by repeated evaporation with absolute alcohol. The residue was then extracted four times with absolute alcohol and the solution, after it had been filtered, was concentrated under reduced pressure, transferred to a dish, and dried in a vacuum desiccator to a syrupy consistency. The syrup, on treatment with absolute alcohol, did not dissolve completely, a small amount of a brown insoluble powder remaining. The alcoholic solution was evaporated to dryness, leaving a thick dark colored syrupy residue.

The following amounts of water-soluble constituents were obtained: In the first analysis, 0.7 gm. of alcohol-insoluble powder and 1.3 gm. of alcohol-soluble syrup, and in the second analysis the corresponding fractions weighed 0.4 gm. and 0.9 gm. The alcohol-insoluble substance gave a slight reduction when boiled with Fehling's solution, while the Molisch reaction was negative. The syrup, on the other hand, strongly reduced Fehling's solution, but the Molisch reaction was negative and no positive reaction for acrolein could be obtained. Evidently, therefore, glycerol was not present.



*Unsaponifiable Matter*

The unsaponifiable matter was a dark brown oil and was highly unsaturated. The iodine number, determined by the Hanus method (5), was 126. In the determination of the iodine number it was noticed that a black, sticky mass separated from the solution, and this material, which evidently represented an iodine addition compound, was very slightly soluble in chloroform, benzene, glacial acetic acid, or in acetone. The unsaponifiable matter gave no definite sterol color reactions.

*Solid Saturated Fatty Acids. Isolation of Palmitic Acid*

The two lots of solid saturated fatty acids were combined, giving a total weight of 16 gm., and converted into the methyl ester. The ester was isolated, dried, and fractionally distilled in a high vacuum.

The residue from the first distillation weighed 2.4 gm., and this fraction was examined, as will be described later.

The distillates, which weighed 14 gm., were further fractionated four times. The principal portion, 10.7 gm., of the ester came over as a colorless oil with the temperature of the bath at 115–117° and at a pressure of about 0.001 mm. The ester crystallized on standing at room temperature and melted at 28°.

The ester was saponified with alcoholic potassium hydroxide, the acid isolated and crystallized once from methyl alcohol yielding 6.9 gm. of snow-white, irregular, thin, plate-shaped crystals which melted at 60–61°. There was no depression of the melting point when some of the acid was mixed with pure palmitic acid which melted at 61°.

*Titration*—0.5071 gm. of acid dissolved in neutral alcohol, with phenolphthalein as indicator, required 19.70 cc. of 0.1 N alcoholic KOH.

$C_{15}H_{32}O_2$ . Mol. wt. calculated, 256; found, 257

A second fraction of the acid was isolated by concentrating and cooling the mother liquor. The crystals thus obtained weighed 1.3 gm., melted at 58–59°, and the molecular weight determined by titration was 258.

The results indicate that the main component of the solid saturated fatty acid fraction is palmitic acid.

*Examination of Higher Boiling Fractions of the Ester*

The higher boiling fractions of the ester obtained in the purification of the methyl palmitate were combined, and, since their melting points were lower than  $28^{\circ}$ , it appeared probable that they were mixtures. The material was therefore saponified and the free acid, after it had been isolated, weighed 3 gm. The acid was converted into the lead salt and the latter was treated with ether. The ether-soluble and ether-insoluble lead salts were decomposed in the usual manner, and we obtained 1.5 gm. of solid acids and 1.4 gm. of liquid acids. Evidently, in the original lead soap-ether separation of the solid and liquid fatty acids, the ether-insoluble lead soap had not been sufficiently washed with ether.

The solid acid fraction was converted into the methyl ester and distilled in a high vacuum. The distillate which came over between  $112-115^{\circ}$  at 0.001 mm. pressure was a colorless oil weighing 1 gm. This fraction was saponified, the free acid was isolated and recrystallized from methyl alcohol. The crystals weighed 0.7 gm. and melted at  $60^{\circ}$ . The molecular weight determined by titration was 259. It is evident, therefore, that this fraction is palmitic acid.

The residue in the distillation flask, weighing about 0.5 gm., was saponified and the free acid isolated and recrystallized three times from methyl alcohol. The crystals thus obtained weighed only 160 mg. and melted at  $63-65^{\circ}$ . The molecular weight determined by titration was found to be 287.

We may conclude that the solid saturated fatty acid fraction consisted principally of palmitic acid. A very small amount of some higher acid, possibly stearic acid, is undoubtedly present, but this acid could not be isolated in a pure state.

*Examination of Residue from First Distillation of Esters of Solid Fatty Acids*

The residue in the distillation flask from the first fractionation of the methyl ester was a brownish solid weighing 2.4 gm., and this fraction should contain the esters of any higher fatty acids which might be present. The substance was saponified with alcoholic

potassium hydroxide and the solution, after having been diluted with water, was extracted several times with ether. The ethereal extract was washed with water, dried with sodium sulfate, filtered, and concentrated to dryness, when a trace of an oily residue was obtained which was discarded. The soap solution was acidified with hydrochloric acid, extracted with ether, and the ethereal extract washed with water until the washings were neutral in reaction. The ethereal solution was then treated with norit, filtered, and the ether distilled off. The residue was a faintly yellowish oil weighing 2.2 gm. which solidified to a wax-like mass on cooling. The substance dissolved in warm acetone or methyl alcohol, but, on cooling, only small globular particles separated. For purification the substance was precipitated by cooling, once from methyl alcohol, twice from a mixture of ether and methyl alcohol, and finally once from acetone, when a snow-white amorphous powder was obtained which weighed about 0.4 gm.

The substance melted at 60–61°. It was a saturated compound because in carbon tetrachloride solution it did not absorb any bromine. The molecular weight determined by titration in alcoholic solution was 488. The substance was optically active.

*Rotation*—0.3669 gm. of substance dissolved in chloroform and made up to 10 cc. gave in a 1 dm. tube a reading of +0.177°; hence,  $[\alpha]_D^{23} = +4.82^\circ$ .

The compound is probably not pure, but it evidently contains a higher hydroxy acid. When treated in pyridine solution with acetic anhydride, an acetyl derivative was formed which melted at 48°. After the acetyl derivative had been saponified with alcoholic potassium hydroxide, the solution was acidified with phosphoric acid and distilled until the distillate was practically neutral in reaction. The amount of acetic acid, as determined by titrating the distillates, was small, being only 5.62 per cent of the substance.

#### *Reduced Fatty Acids*

The reduced fatty acids were purified by recrystallization from methyl alcohol and acetone, but the product was evidently a mixture which could not be separated by crystallization. The melting point of the crystals was 56–57° and the molecular weight was 267.

The remaining portions of the reduced acids were therefore combined, total weight 8.6 gm., and converted into the methyl ester. The ester was fractionated at a pressure of about 0.001 mm. Fraction 1 was collected with the temperature of the bath at 110–112°; Fraction 2, between 115–125°; while Fraction 3 remained as a residue in the distilling flask.

Fraction 1, 3.1 gm., formed a white crystalline mass which melted at 28–29°. The ester was saponified and the free acid isolated and recrystallized once from methyl alcohol and twice from acetone. Snow-white, thin, irregular, plate-shaped crystals were obtained, which weighed about 1.0 gm. The acid melted at 63°, solidified at 60°, and remelted at 63°. There was no depression of the melting point when some of the substance was mixed with pure palmitic acid.

*Titration*—0.3695 gm. of the acid, dissolved in neutral alcohol, with phenolphthalein as indicator, required 14.34 cc. of 0.1 N alcoholic KOH.

$C_{18}H_{32}O_2$ . Mol. wt. calculated, 256; found, 257.6

A second fraction of the acid, recovered from the mother liquor, melted at 61–62°, and the molecular weight, determined by titration, was 259.

These values would indicate that the acid was practically pure palmitic acid.

Fraction 2 of the ester was saponified, the free acid isolated and crystallized from methyl alcohol, yielding 0.8 gm. of colorless, thin, plate-shaped crystals. The substance melted at 60–62° and the molecular weight, determined by titration, was 259.6.

It is evident, therefore, that this fraction also consisted of nearly pure palmitic acid.

#### *Examination of Fraction 3, the Residue in the Distilling Flask*

The residue in the distilling flask, weighing 4 gm., was saponified, the free acid isolated, and recrystallized from methyl alcohol, yielding 1.8 gm. of an acid which melted at 54–55° and which, on titration, was found to have a molecular weight of 282. The substance was recrystallized seven times from acetone and then melted at 67°. After three further recrystallizations from acetone, the substance weighed 0.3 gm. It melted at 68–69°, solidified at

65°, and remelted at 68–69°. Mixed with stearic acid, m.p. 70–71°, it melted at 69–70°, solidified at 66–65°, and remelted at 69–70°.

*Titration*—0.2897 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 9.82 cc. of 0.1 N alcoholic KOH. Found: mol. wt., 295.

*Analysis*—3.028 mg. substance: 3.37 mg. H<sub>2</sub>O and 8.425 mg. CO<sub>2</sub>  
Found. C 75.88, H 12.45

This substance resembles stearic acid in composition and melting point, but the molecular weight is somewhat too high, and it also differs from stearic acid in its crystal habits. The acid always separated from methyl alcohol or acetone in small dense particles which showed no definite crystalline structure. It is not an hydroxy acid because it did not form an acetyl derivative when treated with acetic anhydride in pyridine solution. We are inclined to believe that it is an impure specimen of an acid of higher molecular weight than stearic acid.

The fatty acids contained in the mother liquors were recovered on evaporation of the solvent and converted into lead soaps. The latter were treated with ether and separated into ether-soluble and ether-insoluble fractions which in turn were decomposed in the usual manner with dilute hydrochloric acid. From the ether-soluble fraction we obtained 0.6 gm. of a liquid fatty acid which had evidently escaped being removed in the original lead soap-ether treatment of the reduced acids.

The solid fatty acids obtained from the ether-insoluble lead soap were converted into the methyl ester. The ester was isolated and distilled slowly in a high vacuum. The distillate, which weighed 1.4 gm., came over at a bath temperature of 115° and at a pressure of 0.001 mm. It was a snow-white solid at room temperature and melted at 27°. The ester was saponified and the free acid isolated and recrystallized from methyl alcohol, when snow-white crystals, weighing 0.96 gm., were obtained, which melted at 59–60°. The molecular weight, determined by titration, was 258. The properties indicate that the substance was palmitic acid.

The residue in the distillation flask, which weighed 0.5 gm., was saponified. The acid was isolated and recrystallized seven times from acetone, when about 0.2 gm. of a substance was obtained

which resembled the impure higher fatty acid previously described. The substance melted at 66–67° and the molecular weight was 289.8.

The results obtained in the study of the reduced fatty acid fraction indicate that it consisted principally of palmitic acid. A small amount of a higher acid was present, but could not be identified and no pure stearic acid could be found in this mixture.

#### *Examination of Liquid Saturated Fatty Acids*

The crude liquid saturated fatty acids were combined, converted into the lead soaps, and the latter were dried and extracted with ether. A small amount of ether-insoluble lead salt was filtered off and decomposed with hydrochloric acid when 1.2 gm. of a solid fatty acid were obtained. The acid, after it had been recrystallized from a little acetone, melted at 57–59° and had a molecular weight of 259. This fraction is, therefore, similar to the main portion of the solid reduced acid and evidently had been carried over into the liquid saturated acids in the lead soap-ether separation.

The liquid saturated fatty acid which was isolated from the ether-soluble lead salt was converted into the methyl ester and the latter fractionated seven times at a pressure of about 0.001 mm. During the distillations, two traps, cooled with a mixture of solid carbon dioxide and alcohol, were inserted between the receiver and the vacuum line. In the early fractionations the first trap was found to contain a trace of more volatile oil, but the amount was extremely small and no effort was made to examine this material.

The purest portion of the ester distilled over when the temperature of the bath was 125–126°. The ester was a colorless mobile oil, which solidified when cooled in a freezing mixture of ice and salt, and melted at –5°. The density at 22.5° was 0.8681 and the refractive index at 25° was 1.4416.

*Analysis*—0.0924 gm. substance: 0.1081 gm. H<sub>2</sub>O and 0.2588 gm. CO<sub>2</sub>  
C<sub>18</sub>H<sub>36</sub>O<sub>2</sub> (298). Calculated. C 76.50, H 12.75  
Found. “ 76.38, “ 13.09

The ester was saponified and the free acid isolated and dried in a vacuum desiccator. The acid was a colorless mobile oil which solidified when cooled in ice water and melted at 9–11°. The

refractive index at 25° was 1.4490. The acid was optically inactive.

*Analysis*—0.1003 gm. substance: 0.1149 gm. H<sub>2</sub>O and 0.2783 gm. CO<sub>2</sub>  
C<sub>18</sub>H<sub>36</sub>O<sub>2</sub> (284). Calculated. C 76.05, H 12.67  
Found. “ 75.67, “ 12.82

*Titration*—0.4291 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 14.94 cc. of 0.1 N alcoholic KOH.

C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. Mol. wt. calculated, 284; found, 287.2

The values found are in agreement with the calculated composition of an isomer of stearic acid. The acid appears to be identical with the tuberculostearic acid which has previously been isolated from the acetone-soluble fat of the human tubercle bacillus.

Intermediate fractions of the ester were also saponified and the corresponding acids isolated and analyzed. The composition of these fractions was always found to agree closely with the calculated values for an acid of the formula C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>, although the molecular weight, as determined by titration, was about 300.

If any higher liquid saturated fatty acids were present, one would expect that their esters would be less volatile, and hence they should accumulate in the residues left in the distilling flasks. The residues were therefore saponified and the free acids examined. The acids from these fractions were nearly colorless or slightly yellowish oils which solidified on cooling in ice water and melted at 9–10°. The values for the molecular weight were somewhat higher than required for stearic acid, namely from 305 to 312, but the composition, as determined by combustion, was in agreement with the formula C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. These fractions were optically inactive. It is not impossible that some higher liquid saturated fatty acid is present, but the amount must be quite small and it was impossible to isolate any such acid in a pure condition.

#### SUMMARY

1. The acetone-soluble fat from the timothy bacillus is not a true glyceride because no glycerol could be isolated from the water-soluble components. The fatty acids are apparently combined with some substance other than glycerol, but the nature of this substance could not be determined.

2. The unsaponifiable matter is an unsaturated oil which gives no sterol color reactions.

3. The fat contains traces of volatile fatty acids.

4. The solid saturated fatty acids consist principally of palmitic acid. Traces of higher fatty acids were found, but no pure stearic acid could be isolated. A small amount of a higher optically active hydroxy acid is also contained in this fraction.

5. The unsaturated fatty acids belong principally to the  $C_{16}$  series, because palmitic acid is formed on catalytic reduction. Traces of higher acids are present in this fraction.

6. The principal constituent of the liquid fatty acids is a saturated fatty acid isomeric with stearic acid,  $C_{18}H_{36}O_2$ , which is similar to tuberculostearic acid.

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## A REDETERMINATION OF THE OXIDATION POTENTIAL OF THE HEMOGLOBIN-METHEMOGLOBIN SYSTEM

BY JAMES B. CONANT AND ALWIN M. PAPPENHEIMER, Jr.

(From the Converse Memorial Laboratory of Harvard University, Cambridge)

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The previous determinations of the oxidation-reduction potential of hemoglobin have not been entirely satisfactory. The direct electrochemical method (1) yielded results uncertain by  $\pm 20$  millivolts whose mean differed by 25 millivolts from the value obtained by an indirect spectrophotometric procedure (2). It seemed worth while, therefore, to attempt once again the electrochemical determination of the oxidation-reduction potential of hemoglobin, with crystalline hemoglobin and methemoglobin. A convenient method for purifying the latter substance has been recently perfected in this laboratory (3).

### *Apparatus*

The glass titration vessel of 50 cc. capacity was connected with a calomel cell through a stop-cock and a tube projecting from the bottom containing saturated potassium chloride and agar, and closed at the extreme end with a ground glass stopper lubricated with the same mixture. A similar arrangement has been used previously in this laboratory in connection with our work on non-aqueous solvents. (A diagram of such a cell is given in (4), p. 4449.) In making a measurement the stop-cock was opened, which lowered the resistance to a small value. The top of the cell was closed by a large rubber stopper which carried the electrodes, inlet and outlet tubes for gases, two burettes protected against oxygen, and the stirrer. The latter was of glass attached to a metal shaft running through a metal "stuffing box" which enabled one to maintain a vacuum satisfactorily even when the stirrer was rotated by a motor. With this stirrer and with the stop-cock closed, it was possible to remove rapidly all oxygen from the cell

and its contents by evacuation and admission of purified nitrogen. In a typical experiment, the cell is evacuated and filled with oxygen-free nitrogen five times, after which no appreciable amount of oxygen is left in the system. Indigotetrasulfonate, titrated just to its end-point, did not become colored in the vessel, even when stirred overnight.

The previous difficulties in using the direct electrochemical method had appeared to rest mainly with the electrodes. Electrodes used in hemoglobin solutions were found to "poison" extremely easily and were easily polarized. In addition, they tended to become sluggish and often took several hours to come to equilibrium. In the experiments here described, a Compton electrometer with a 1,000,000 ohm resistance in series with the cell was used in order to prevent polarization of the electrodes. A motor stirrer was fitted into the cell which kept the solutions continually flowing past the electrodes. This appeared in some cases to shorten the time for obtaining equilibrium. In addition to bright platinum foil, burnt-on platinum electrodes were tried after the method of Westhaver (5). For hemoglobin, these latter behaved better in general, being much quicker to come to equilibrium, although they were more easily poisoned. Burnt-on electrodes do not become polarized to any great extent even without the use of the electrometer.

#### *Preparation of Hemoglobin and Methemoglobin*

Hemoglobin was prepared by the method of Ferry and Green (6). A typical preparation was as follows: About 3 liters of horse plasma residues were washed with 12 liters of isotonic NaCl (0.9 per cent) and run through a Sharples centrifuge which was kindly put at our disposal by Dr. E. J. Cohn and Mr. Bennett. 3 liters of laked concentrated hemoglobin solution were recovered. 0.1 N hydrochloric acid was then added with stirring in the cold room until the appearance of platelets. About 400 to 500 cc. of acid were necessary. Stirring was usually continued overnight. The crystalline mixture was then centrifuged and the paste washed twice with equal volumes of iced distilled water. The yield of washed paste was 1700 to 1800 gm. The crystalline paste (1100 cc.) was treated with 0.990 N potassium hydroxide until solution was complete. The solution was centrifuged and the clear concentrated supernatant liquid poured off from a small amount of

undissolved residue. The pH was then adjusted to 6.6 and the mixture was stirred 5 hours until crystallization was complete, and then centrifuged, washed once with distilled water, and once with phosphate buffer of pH 7.0. About 100 cc. of this recrystallized paste were dissolved with stirring in the phosphate buffer of pH 7.0 and ionic strength 0.24. When the oxyhemoglobin had all dissolved, the solution was centrifuged and the supernatant solution poured off into a 1 liter tonometer. The oxygen was pumped off by repeated evacuation and filling with nitrogen. It took usually 2 to 3 days to get the oxygen completely removed. During this time some methemoglobin always formed, which was determined by analysis and corrected for in the calculations.

The methemoglobin (prepared according to Levy) was recrystallized by dissolving in KOH and then adding the calculated amount of acid. In some experiments the methemoglobin was not recrystallized but only washed three times with water. Occasionally difficulty was had in getting the methemoglobin to crystallize out of a supersaturated solution. In this case it may be necessary to continue stirring for a whole day. Stirring at room temperature for a short time seems to accelerate crystallization. The entire preparation of hemoglobin and methemoglobin was carried out in the cold room at 0°. Analyses showed that bacterial action was inappreciable after the solutions had been stored 2 months at this temperature. However, solutions could not be safely kept more than 1 day in phosphate buffers in the titrating apparatus at room temperature.

The solutions of hemoglobin and methemoglobin were analyzed by the Van Slyke method (7) for hemoglobin and for methemoglobin and cathemoglobin according to the methods developed in this laboratory (8). The amount of cathemoglobin was less than 0.5 per cent of the total hemoglobin (or methemoglobin) in the stock solutions, which was 6 to 8 per cent. The amount of methemoglobin in the hemoglobin solutions was 18 per cent of the total hemoglobin and this amount was taken into account in calculating the ratio of hemoglobin to methemoglobin in the cell.

#### *Procedure*

The method employed was that known as the method of mixtures. The hemoglobin and methemoglobin solutions were placed in special containers connected to the two burettes so arranged

that no oxygen would have access to the solutions. Oxygen-free nitrogen was used to force the liquids into the burettes. It was found advantageous to add to the liquid in the titration cell an amount of potassium ferrocyanide equivalent to half the hemoglobin present; this greatly improved the electrode equilibrium. The phosphate buffer solution was placed in the cell, freed from air by repeated evacuation and introducing nitrogen. Definite amounts of the two stock hemoglobin solutions were then added and the electrical readings made. In the most satisfactory runs equilibrium was attained at each point after about half an hour, and the change during even this time was not more than 4 millivolts; in other runs it seemed impossible to obtain satisfactory electrode equilibrium, and the results had to be discarded. There is no doubt that the electrodes are very susceptible to poisoning in the determinations of the hemoglobin potential, and a great number of experiments may have to be performed before reproducible satisfactory results are obtained.

### Results

The results plotted in Fig. 1 are the best which we have been able to obtain. The points represent different ratios of hemoglobin and methemoglobin obtained in one experiment, by introducing into the cell 12.5 cc. of stock methemoglobin solution and adding increments first of hemoglobin solution and then of methemoglobin solution. At the mid-point the concentrations of hemoglobin and methemoglobin were 3.5 milli-equivalents per liter,  $\mu = 0.3$  and  $\text{pH} = 7.0$ ; the temperature was  $24^\circ$ . The value of  $E_0$  was found to be  $152 \pm 5$  millivolts, which agrees excellently with the value of 0.150 volt found by the spectrophotometric measurements at  $\text{pH} = 6.9$  and  $\mu = 0.5$ . The best straight line has been drawn through the points; the slope of this line cor-

$$E = E_0 + \frac{RT}{n} \ln \frac{[\text{MHb}]}{[\text{Hb}]}$$

responds to a value of  $n$  in the usual electrochemical equation of 1.2. It is evident that this one experiment is as satisfactory as one could desire in regard to the consistency of the results; it might be mentioned, further, that over the first half of the curve both electrodes agreed within 3 millivolts, but one electrode became

poisoned half-way through the experiment and gave no readings thereafter.

A number of experiments with a buffer solution of  $\mu = 1.6$ , pH = 6.95, and a concentration of hemoglobin and methemoglobin of 1.4 milli-equivalents per liter at the mid-point gave less consistent results. The value of  $E_0$  (at 24°) ranged from 0.117 to 0.136 volt with an average of 0.129. These results indicate clearly that a large increase of the ionic strength (from  $\mu = 0.3$  to  $\mu = 1.6$ ) decreases the oxidation-reduction potential somewhat. When all these results are plotted in a way similar to that shown in

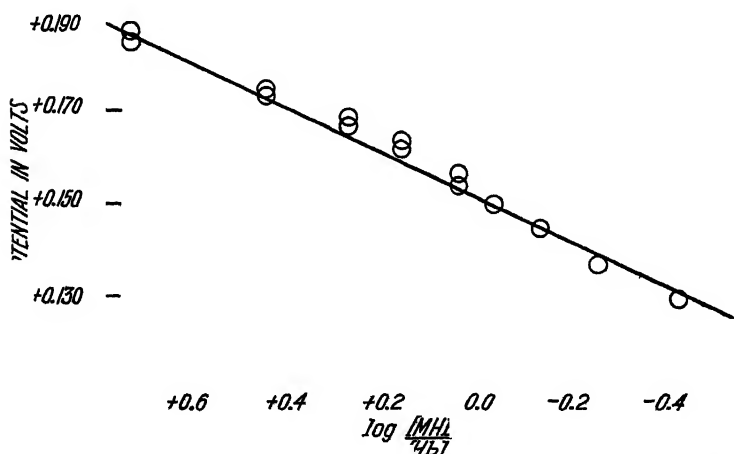


FIG. 1. Determination of oxidation-reduction potential of hemoglobin by method of mixtures at 24°, pH = 7.0,  $\mu = 0.3$ .

Fig. 1 and the best line drawn through them, it was found to have a slope corresponding to  $n = 1$ , though in a number of cases a slope corresponding to a value of  $n = 2$  was indicated.

The total concentration of the hemoglobin seems to be without appreciable effect on the potential or the value of  $n$  if adequate buffers are employed. Thus three experiments with a concentration of only 0.023 milli-equivalents per liter (at the mid-point) in a buffer solution of  $\mu = 1.6$ , pH = 6.95, gave a value of  $E_0$  of 0.131 to 0.146 and a value of  $n$  of about 2. In this connection it should be noted that the spectrophotometric measurements were made with a total hemoglobin concentration of about 0.06 milli-equivalents per liter.

The data plotted in Fig. 1 make it evident that under favorable circumstances the oxidation-reduction potential of hemoglobin may be determined with assurance. Unfortunately, no conclusive information can be obtained, however, about the value of  $n$ . Values less than 1 and greater than 2.5 are clearly excluded both in concentrated and dilute solutions of hemoglobin; this is in accord with the results of the spectrophotometric study. Therefore, the oxidation of hemoglobin ( $\text{Hb}_4 = \text{mol. wt. } 66,000$ ) cannot be represented by the simple equation,  $\text{Hb}_4 - 4e \rightarrow \text{MHb}_4$ , unless some auxiliary assumptions are made. The problem is exactly like that presented by the oxygenation of hemoglobin; the assumption of intermediate compounds can be used also as an explanation of the abnormal value of  $n$  in the transformation of hemoglobin to methemoglobin. The failure of experiments in this laboratory (9) designed to obtain evidence in favor of this view cannot be considered as conclusive negative evidence, of course. However, until direct evidence is available to support this explanation, it seems important to emphasize that the quantitative aspects of the problem of the oxygenation and oxidation of hemoglobin still remain unsolved.

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## THE SPECTROPHOTOMETRIC DETERMINATION OF CERTAIN BLOOD PIGMENTS

By G. B. RAY, H. A. BLAIR, AND C. I. THOMAS

*(From the Department of Physiology, School of Medicine, Western Reserve University, Cleveland)*

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The use of the spectrophotometer as a means of estimating the relative and total concentrations of blood pigments was first introduced by Hüfner (1, 2), following the theoretical work of Vierordt (3). This author published tables whereby the relative concentrations of oxyhemoglobin, hemoglobin, carboxyhemoglobin, and methemoglobin could be estimated from the quantitative character of the absorption curve. Since Hüfner's original papers, several investigators in this field have made redeterminations of his constants for pigments in pure solution but no revision of his tables has been carried out. It is of the greatest convenience to have these tables available for they offer an accurate and rapid means of estimating the relative concentration of any two pigments. The data given in this paper represent an adaptation of Hüfner's method to more recently determined constants and a simplification of the technique of computation. By means of simple formulæ or previously prepared charts it is possible to estimate the concentration of total pigment, oxyhemoglobin, and a second pigment on as small a sample of blood as 0.05 cc. in a very short time with a high degree of precision. This method is also applicable to any sort of colored solution; for example, indicators. One may thus apply the same theory to the data given by Brode (4) and thereby estimate the hydrogen ion concentration of a solution (5).

The accuracy of an oxyhemoglobin determination by this means is, according to Davis and Sheard (6), comparable to that of the gasometric method with the constant volume Van Slyke apparatus in the case of normal bloods or pigment concentrations. In the case



of very dilute solutions, the accuracy of the spectrophotometer is unchanged, while the error of the gasometric method is increased. A second advantage, perhaps the most important, of the spectrophotometric method is the rapidity and ease with which one may make determinations. A determination of hemoglobin concentration can be made with check analyses in the time needed for a single gasometric reading.

Furthermore, it is possible to check the purity of the pigment at the time of the determination. While the gasometric determination gives only the functional hemoglobin, one may, by means of the spectrophotometer, recognize at once the presence of a contaminating substance. Thus, in solutions of purified hemoglobin, where methemoglobin formation may occur spontaneously, any deviation from the normal relationships may be detected at once and the extent of inactivation calculated.

### *Theoretical*

Lambert's law expresses the fact that in an absorbing medium the rate of loss of intensity of a light beam penetrating the medium varies at any given point as the intensity at that point; *i.e.*, at any point

$$\frac{dI}{dn} = -kI \quad (1)$$

where  $n$  is the distance measured through the medium,  $I$  the light intensity, and  $k$  a constant called the absorption coefficient or transmissive exponent.

By integrating and letting the incident intensity be  $I_0$ , we obtain

$$\log I - \log I_0 = -kn$$

or

$$-\log T = kn$$

where  $T$  is the fraction of the light transmitted, which quantity is called the transmission.

In practice it is convenient to use logarithms to the base 10 giving

$$-\log_{10} T = En \quad (2)$$

where  $E$  is the transmissive index or extinction coefficient.

When Equation 1 is divided by  $I$ , it will be seen that  $k$  is the rate of decrease of intensity per unit intensity. Since  $E$  is proportional to  $k$ , it has a similar meaning. It is clear that the more absorbing the medium the greater will be the rate of diminution of the intensity; in particular, in solutions,  $E$  will be greater the more concentrated the solution.

Beer's law expresses the fact that for any given substance in solution,  $E$  varies directly as the concentration, or

$$E = \frac{1}{A} \cdot c \quad (3)$$

where  $\frac{1}{A}$  is a constant and  $c$  the concentration.<sup>1</sup> It is evident from Equation 3 that  $\frac{1}{A}$  is the  $E$  or extinction coefficient for a unit concentration of the substance. In practice, use is made of  $A$  rather than  $\frac{1}{A}$  and it is called the absorption ratio. Since the  $A$  values of different substances are in general different, they may be used to identify or distinguish their respective substances. The  $A$  value of a substance is thus an important specific measure of the properties of the substance. The method of its determination may now be considered.

From Equations 2 and 3 we deduce that

$$-\log_{10} T = \frac{c}{A} n \quad (4)$$

which gives a means of determining  $A$  if the concentration of the solution and its length are known.

The fact has been neglected thus far that the absorption is different for lights of different wave-lengths. This being taken into consideration, it is evidently necessary to specify the wave-length at which  $A$  is measured to give at wave-length  $\lambda$  the relation

$$-\log T_{\lambda} = \frac{c}{A_{\lambda}} n \quad (5)$$

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<sup>1</sup> Unpublished data show that Beer's law does not hold over an extreme range of concentration in the case of hemoglobin. It does hold, however, for concentrations usually employed in the spectrophotometry of the blood pigments.

which provides a means for determining  $A_\lambda$ . It should be observed that the numerical value of  $A_\lambda$  depends on the units used for  $c$  and  $n$ . The usual procedure is to express  $n$  in cm. and  $c$  in gm. per cc. For example, when this is done for oxyhemoglobin at wave-lengths  $540\ m\mu$  and  $560\ m\mu$  respectively the following  $A$  values are obtained.

$$\begin{aligned} A_{540} &= A = 0.0011 \text{ (Davis and Sheard)} \\ A_{560} &= A' = 0.00178 \end{aligned} \quad (6)$$

It will be seen from Equation 5 that if  $n = 1$  and the transmission  $T = \frac{1}{10}$  then

$$1 = \frac{c}{A} \quad (7)$$

It is thus evident that  $A$  is numerically equal to the concentration (in gm. per cc.) necessary to reduce the incident light to one-tenth its intensity after passing through 1 cm. of solution. This provides a convenient picture of  $A$ . By referring to Equation 6, it is seen that 0.0011 gm. per cc. of oxyhemoglobin reduces light of wave-length  $540\ m\mu$  to one-tenth its incident intensity after passing through a length of 1 cm. of solution but at wave-length  $560\ m\mu$  0.00178 gm. per cc. is required to produce the same result.

By using  $T$  and  $A$  and  $T'$  and  $A'$  for  $T$  and  $A$  at  $540\ m\mu$  and  $560\ m\mu$  respectively, there are obtained from Equation 5 the two equations

$$-\log T = \frac{c}{A} n \quad (8)$$

$$-\log T' = \frac{c}{A'} n \quad (9)$$

When Equation 8 is divided by Equation 9

$$(10)$$

This ratio, since  $c$  and  $n$  have dropped out, is independent of the concentration and the length of the tube. It is a constant then for any given substance whose value can be obtained by measuring

only  $T$  and  $T'$ . It will be hereafter designated by  $R$ . It is usually called Hüfner's ratio.

From Equations 3 and 10 is derived the equation

$$R = \frac{A'}{A} = \frac{E}{E'} \quad (11)$$

Therefore, these ratios are interchangeable. For example, their numerical value for oxyhemoglobin is, according to Equation 6,

$$R = \frac{0.00178}{0.0011} = 1.619 \quad (12)$$

$R$ , since it involves two  $A$  values, is evidently a much more specific identification for a particular substance than is a single  $A$  value.

The considerations thus far give a means of identifying a substance in solution (Equation 10) when its concentration is not known or of estimating its concentration (Equation 8 or 9) when its identity is known. But no means have been provided for estimating the components of a mixture. This may be done easily for a mixture of two substances in the following way.

Since each of the substances will absorb just as if it were alone in solution, Lambert's law may in this case be written

$$\frac{dI}{dn} = -kI - k_1I$$

where the  $k$  values are the transmissive exponents of the first and second substances respectively.

By integrating as before, we obtain

$$-\log \frac{I}{I_0} = (k + k_1) n$$

or

$$-\log_{10} T = (E + E_1) n \quad (13)$$

where  $E$  and  $E_1$  are again the respective extinction coefficients of the first and second substances.

It is convenient at this point to introduce the idea,

$$E_{\text{mix.}} = E + E_1 \quad (14)$$

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in which  $E_{\text{mix.}}$  is the extinction coefficient of the mixture, or by use of Equation 3 to obtain

$$\frac{c_{\text{mix.}}}{A_{\text{mix.}}} = \frac{c}{A} + \frac{c_1}{A_1} \quad (15)$$

$$A_{\text{mix.}} = c_{\text{mix.}} A + \frac{c}{c_{\text{mix.}}} A_1$$

or if the fraction  $\frac{c}{c_{\text{mix.}}}$  of the first substance be represented by  $x$  and the fraction  $\frac{c_1}{c_{\text{mix.}}}$  of the second by  $y$  then

$$\frac{1}{A_{\text{mix.}}} = \frac{x}{A} + \frac{y}{A_1}$$

$$\text{or} \quad A_{\text{mix.}} = \frac{AA_1}{xA_1 + yA} \quad (16)$$

Thus, if  $A$  and  $A_1$  are known,  $A_{\text{mix.}}$  can be calculated for any mixture containing  $x$  parts of the first ( $A$ ) substance and  $y$  parts of the second ( $A_1$ ) substance.

From Equations 13, 14, and 15 is derived the equation

$$-\log_{10} T = E_{\text{mix.}} n = \frac{c_{\text{mix.}} n}{A_{\text{mix.}}} \quad (17)$$

which provides a means of obtaining the total concentration of a mixture when  $A_{\text{mix.}}$  has been found.

Since  $x$  and  $y$  must be known to determine  $A_{\text{mix.}}$  it is now necessary to consider how these may be obtained. This may be done by measuring the light transmitted by the mixture at two different wave-lengths; for, by use of Equations 15 and 17 are obtained the equations

$$-\log T = \frac{c_{\text{mix.}} n}{A_{\text{mix.}}} = \left( \frac{c}{A} + \frac{c_1}{A_1} \right) n \quad (18)$$

$$-\log T' = \frac{c_{\text{mix.}} n}{A'_{\text{mix.}}} = \left( \frac{c}{A'} + \frac{c_1}{A'_1} \right) n \quad (19)$$

in which the letters that do not have the prime apply to the first wave-length ( $540m\mu$ ) and the primed letters apply to the second wave-length ( $560m\mu$ ). When Equation 18 is divided by Equation 19 and  $R$  is considered the ratio of the logarithms, the resulting equation is

$$R = \frac{\log T}{\log T'} = \frac{A_1c + Ac_1}{AA_1} \cdot \frac{A'A'_1}{A'_1c + Ac_1} \quad (20)$$

This equation shows that  $R$  is independent of the absolute concentration, for, if the  $c$  values on the right-hand side are all multiplied by the same factor,  $R$  remains unchanged.

By dividing the numerator and the denominator by the total concentration,  $c_{\text{mix.}} = c + c_1$ , and by assuming that

$$\frac{c}{c_{\text{mix.}}} = x \text{ and } \frac{c'_1}{c_{\text{mix.}}} = y = 1 - x$$

we obtain the equation

$$R = \frac{A_1x + A(1-x)}{AA_1} \cdot \frac{A'A'_1}{A'_1x + A'(1-x)} \quad (21)$$

which provides a means of determining  $x$  and then also  $y$  when  $R$  has been measured.  $x$  and  $y$  having been determined in this way,  $A_{\text{mix.}}$  can be obtained from Equation 16 and then the total concentration of pigment in the mixture can be obtained by use of Equation 17. It should be observed in doing this, that the same wave-length must be used for Equation 17 as that for which  $A_{\text{mix.}}$  is calculated in Equation 16; *i.e.*, the wave-length to which  $A$  and  $A_1$  apply.

It will be remembered (Equation 3) that  $\frac{1}{A}$  is a more natural unit than  $A$  itself, but the argument has been carried through with  $A$  values because they are commonly used. Greater simplicity is attained in Equation 21, however, by using units of  $\frac{1}{A}$  which may be written  $r$ , so that

$$r = \frac{1}{A}, r_1 = \frac{1}{A_1}, \text{ etc.}$$

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Equations 18 to 21 written as Equations 18, *a* to 21, *a* when  $\frac{1}{A}$  is replaced by *r*, become

$$-\log T = (cr + c_1r_1)n \quad (18,a)$$

$$-\log T' = (cr' + c_1r'_1)n \quad (19,a)$$

$$R = \frac{cr + c_1r_1}{cr' + c_1r'_1} \quad (20,a)$$

$$R = \frac{xr + (1-x)r_1}{xr' + (1-x)r'_1} \quad (21,a)$$

From these is obtained Equation 22

$$\frac{Rr'_1 + r_1}{R(r' - r'_1) + (r_1 - r)} \quad (22)$$

which is easier to use than Equation 21. If *x* is the required per cent, Equation 22 may be multiplied by 100, in which case *y* = 100 - *x* instead of 1 - *x*.

### EXPERIMENTAL

The theoretical manipulation of Lambert's law in the preceding section shows clearly that if the various derivations are to be of practical use it is necessary to establish certain constants and to introduce calculations involving these constants. The data in this paper, except where otherwise noted, were obtained with the Keuffel and Esser color analyzer (7). For a general discussion of spectrophotometry and spectrophotometers the reader is referred to the "Report of the Committee on Spectrophotometry of the Optical Society of America, 1922-23" (8).

In the data that follow, only fresh bloods were used unless otherwise noted and 0.4 per cent ammonium hydroxide was used as a diluent. All readings of transmission were made in groups of five and the averages taken. As mentioned above, only two wavelengths need be read to utilize the formulæ; throughout this paper 540*mμ* and 560*mμ* have been used. In some cases a second observer made a second series of readings on a similar sample. All glassware employed was calibrated.

As may be gathered from the derivation of the formulæ, the

most important and fundamental measurement is the ratio value,  $R$ . Until this constant has been evaluated for the various pigments it is impossible to estimate their purity which is of the utmost importance. Furthermore, this represents the most studied phase in regard to the blood pigments, and, unfortunately, the most varied.

In the case of oxyhemoglobin, the generally accepted figure at the present time is that of Hári (9) who found the value of  $R$  to be 1.606. As a result of twenty-five analyses, we find an average value of  $R$  to be 1.619, that is 0.8 per cent higher. This variation is within the error of the measurement when one considers that two different types of photometer were used. The standard deviation of our estimation was 0.038. These results are lower than those found by Charnas (10) on a large number of bloods, but higher than that found by Hüfner (1). The latter gives a value of 1.578 which was also found by Butterfield (11), using the Hüfner spectrophotometer. On the same type of apparatus, Aron and Muller (12) found still lower values. The variation seems to be due to the width of the band studied; for Heubner and Rosenberg (13), using the accurate method of measuring the density of spectrographic plates, found values corresponding to those of Hári and other investigators when a narrow band was measured. Their experiments seem to be crucial in explaining why such low values have been found by various workers, for Hüfner's measurements were made on a band of  $11m\mu$ . No reason can be suggested why the results of Charnas should be higher than those of the others. From the above, however, it must be clear that each instrument should be checked and the value of  $R$  established for that particular instrument.

The value of  $R$  as an average of twenty samples of methemoglobin was found to be 1.225 with a standard deviation of 0.0354. This compares favorably with the 1.215 found by Hári (9) and 1.212 found by Heubner and Rosenberg (13). The variation from Hári's figure is identical with that found for oxyhemoglobin; namely, 0.8 per cent. It would appear, therefore, that the constant variation was a function of the apparatus.

The methemoglobin was produced by oxidizing hemoglobin with potassium ferricyanide or amyl nitrite. The figures given above are for alkaline methemoglobin since the diluent was ammoniacal.



Carboxyhemoglobin, made by saturating the blood with pure CO, gave a value for  $R$  of 1.168 as an average of twenty-five determinations. The standard deviation was 0.0288. For the same pigment Hüfner gives 1.095 as the ratio while Haurowitz (14) finds 1.175.

Reduced hemoglobin was found to have  $R = 0.846$  as opposed to Hüfner's 0.796. Our standard deviation was 0.0118. In our experiments the reduction was carried out by the addition of 1 cc. of ammoniacal 4 per cent sodium hydrosulfite to each 100 cc. of dilution. The reduction was immediate and stable over a period of 2 hours. There was no indication of turbidity due to sulfur precipitation during this period of time.

The evaluation of the ratio  $R$  offers at once a concise and, as may be judged from the magnitude of the standard deviations, accurate method of estimating the purity of the pigment in solution. It should be kept in mind that the ratio is non-specific and gives no information as to the nature of the second pigment, if one be present. Even if the second pigment is known further, data must be developed in order that its relative concentration be estimated. We must, therefore, first establish certain other constants. In the formula given for the ratio of a mixture (Equation 21) it is necessary to know the absorption ratios of the two pigments at the working wave-lengths, namely  $540m\mu$  ( $A$ ) and  $560m\mu$  ( $A'$ ). These can be obtained only when the concentration of each is known. The measurement of the value of  $A_{540}$  is of further importance since it allows us to calculate the concentration of any pure blood pigment in solution.

In the case of oxyhemoglobin,  $A$  has been found to be 0.0011 by Davis and Sheard (6). We have used this value repeatedly and found excellent checks with the Van Slyke apparatus. In the data that follow we have computed the concentration of oxyhemoglobin from the spectrophotometric method and checked the result by the gasometric method. The various pigments were made in the same manner as described above, with great care being taken to insure accurate dilution. The value of  $R$  was checked in each case so that errors due to incomplete action of the reagents might be avoided.

Table I gives the results of this series of measurements. The second column gives the concentrations of pigment as oxyhemo-

globin. These figures are those found by the spectrophotometer but in every case the measurement was confirmed by the Van Slyke apparatus. The remaining data are for the value of the absorption ratio at 540  $m\mu$  calculated from Equation 5. For convenience, only the values of  $A_{540}$  are given in Table I. The value of  $A_{560}$  can be readily computed since by Equation 10

$$\frac{A_{540}}{A_{560}} = R$$

TABLE I

*Values of Absorption Ratio at 540  $M\mu$  for Carboxyhemoglobin, Methemoglobin, and Reduced Hemoglobin*

Determination No.	Hb concentration	$A_{540}$		
		HbCO	MHb	Hb
	<i>per cent</i>			
1	10.96	0.001172	0.00201	0.00160
2	11.14	0.001204	0.00205	0.00160
3	13.12	0.001127	0.00163	0.00152
4	13.26	0.001067	0.00163	0.00152
5	13.44	0.001116	0.00161	0.00153
6	13.34	0.001133	0.00172	0.00152
7	13.20	0.001062	0.00158	0.00150
8	12.92	0.001069	0.00151	0.00149
9	13.42	0.001117	0.00170	0.00155
10	13.29	0.001096	0.00163	0.00150
11	11.25	0.001074	0.00178	0.00148
12	11.15	0.001160	0.00177	0.00151
13	15.09	0.001100	0.00171	0.00150
14	15.36	0.001106	0.00173	0.00150
15	12.73	0.001087	0.00166	0.00148
Mean.....		0.001113	0.001715	0.001520

Therefore, in the case of carboxyhemoglobin, we find the mean value of  $A_{540}$  to be 0.001113 as compared with 0.001175 found by Haurowitz. The value for  $A_{560}$  will, therefore, be 0.001301. This value checked with that found experimentally.

$A_{540}$  for methemoglobin is 0.001715 and  $A_{560}$ , 0.002126. These values are in excellent agreement with those given by Hári (9) who found  $A_{540}$  to be 0.001725 and  $A_{560}$  to be 0.002095.

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For reduced hemoglobin  $A_{540}$  is 0.001520 and  $A_{560}$  is 0.001286. These values are lower than those given by Hüfner (1) ( $A_{540} = 0.001778$  and  $A_{560} = 0.001354$ ) which might be predicted from the discussion of the variations of the value of  $R$  for oxyhemoglobin.

The above data supply the necessary information for the calculation of the concentration of single pigments.

Further data are necessary if one is to determine the total concentration in a mixture of two pigments. Not only must we know

TABLE II  
*Changes in Value of Ratio  $R$  for Mixtures of Oxyhemoglobin with Carboxy-hemoglobin, Methemoglobin, or Reduced Hemoglobin*

HbO <sub>2</sub>	$R$			HbO <sub>2</sub>	$R$		
	HbCO	MHb	Hb		HbCO	MHb	Hb
<i>per cent</i>				<i>per cent</i>			
0	1.168	1.224	0.846	55	1.360	1.457	1.208
5	1.185	1.248	0.874	60	1.403	1.476	1.248
10	1.202	1.270	0.903	65	1.421	1.495	1.288
15	1.219	1.292	0.933	70	1.452	1.513	1.331
20	1.238	1.314	0.964	75	1.477	1.531	1.374
25	1.256	1.336	0.996	80	1.503	1.549	1.420
30	1.275	1.357	1.029	85	1.531	1.567	1.467
35	1.295	1.377	1.062	90	1.559	1.584	1.515
40	1.315	1.398	1.097	95	1.588	1.601	1.566
45	1.336	1.418	1.133	100	1.618	1.618	1.618
50	1.358	1.437	1.170				

the absorption ratio but also the value of  $R$  for any mixture. It will be recalled that in a mixture

$$R = \frac{x r_{540} + (1 - x) r_{560}}{x r'_{540} + (1 - x) r'_{560}}$$

where in the particular case with which we are dealing  $r = \frac{1}{A}$  in which the  $A$  values are the absorption ratios for oxyhemoglobin and the  $A'$  values are the absorption ratios for the second pigment. It is necessary, therefore, to compute the various values for the absorption ratio with variations in the relative concentrations  $x$  and  $y$  and from this changes in the ratio  $R$ .

Table II presents the calculated value of  $R$  for mixtures of either

carboxyhemoglobin, methemoglobin, or reduced hemoglobin with oxyhemoglobin. The first and fifth columns of Table II refer to the relative concentration of the second pigment by increasing amounts of 5 per cent. While Table II may be used to compute the relative concentrations, on the assumption that between any two successive figures the relationship is linear, the authors have found

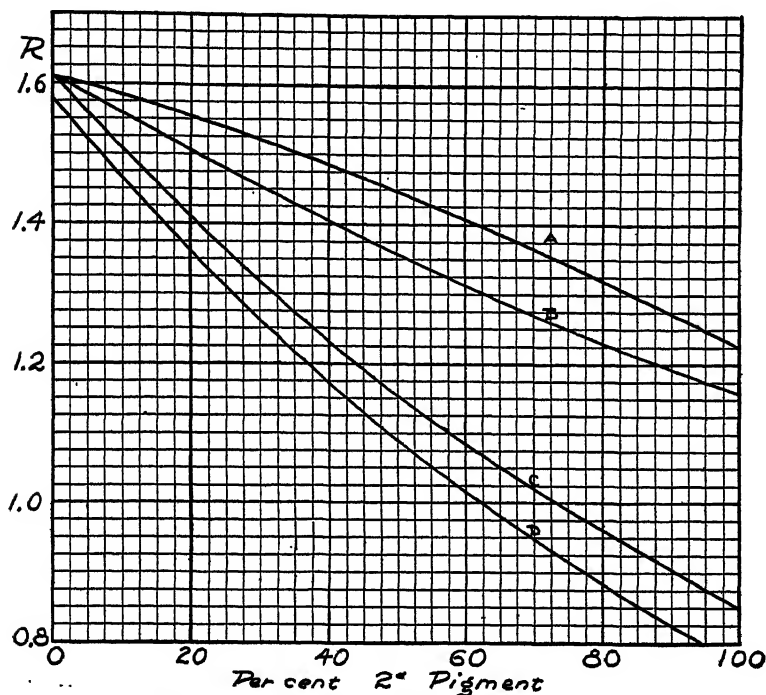


FIG. 1. Changes in the value of the ratio,  $R$ , with decreasing amounts of oxyhemoglobin in the presence of methemoglobin (Curve A), carboxyhemoglobin (Curve B), and reduced hemoglobin (Curve C). Curve D represents Hüfner's (1) results for reduced hemoglobin.

it more desirable to plot  $R$  against concentration in the manner shown in Fig. 1, by use of the data given in Table II. Such a chart, if made with a sufficiently large scale, permits estimation to the fourth significant figure with ease. Attention is directed in Fig. 1 to Curve D which has been given for the purpose of comparison. It is plotted from Hüfner's data for reduced hemoglobin

and it will be observed that the variation is such that the error may be as high as 300 per cent; *i.e.*, when the relative concentration is found to be 5 per cent by the corrected figures the older value indicates 20 per cent.

Once having obtained the relative concentrations of the two pigments, one may then determine the total pigment. By far the simplest means of doing this, for routine work, is to utilize a table or chart of the computed values of  $A$  for any mixture, *i.e.*  $A_{mix.}$ . Such a table is given in Table III and its companion chart in Fig. 2. As mentioned above, either method may be utilized success-

TABLE III  
*Changes in Absorption Ratio ( $A_{mix.}$ ) of a Mixture of Oxyhemoglobin with Carboxyhemoglobin, Methemoglobin, or Reduced Hemoglobin*

HbO <sub>2</sub> per cent	$A_{mix.}$			HbO <sub>2</sub> per cent	$A_{mix.}$		
	HbCO	MHb	Hb		HbCO	MHb	Hb
0	0.001115	0.001715	0.001520	55	0.001106	0.001311	0.001256
5	0.001114	0.001667	0.001491	60	0.001106	0.001284	0.001237
10	0.001113	0.001624	0.001464	65	0.001105	0.001258	0.001218
15	0.001113	0.001582	0.001438	70	0.001104	0.001233	0.001199
20	0.001112	0.001542	0.001412	75	0.001104	0.001208	0.001182
25	0.001111	0.001505	0.001387	80	0.001103	0.001185	0.001164
30	0.001110	0.001469	0.001364	85	0.001102	0.001163	0.001148
35	0.001110	0.001434	0.001341	90	0.001102	0.001141	0.001131
40	0.001110	0.001401	0.001319	95	0.001101	0.001120	0.001115
45	0.001108	0.001371	0.001297	100	0.00110	0.00110	0.00110
50	0.001107	0.001340	0.001276				

fully. Once the absorption ratio for the mixture has been found, it is treated in the same manner as though it were a pure solution.

An optional method may be suggested for the determination of total pigment in oxyhemoglobin solutions containing either carboxy- or methemoglobin. This procedure has the advantage of making possible the detection of the presence of a third pigment and is also recommended in cases where uncertainty exists as to the nature of the second pigment. The method consists, in the case of carboxyhemoglobin, of saturating the whole solution with carbon monoxide and determining the value of  $R$ . Obviously, if the value of  $R$  deviates from 1.168 a third pigment is present. If

the value is that of carboxyhemoglobin the total pigment concentration may be estimated from the usual formula for pure pigments. In the case of methemoglobin a slightly different method must be used; namely, one similar to that used by Van Slyke (15) for the gasometric determination of methemoglobin. The hemoglobin is reduced by the action of ammoniacal sodium hydrosulfite (1 cc. of 4 per cent  $\text{Na}_2\text{S}_2\text{O}_2$  in 2 per cent  $\text{NH}_4\text{OH}$ ) in 100 cc. dilution and the blood is at once saturated with carbon monoxide.

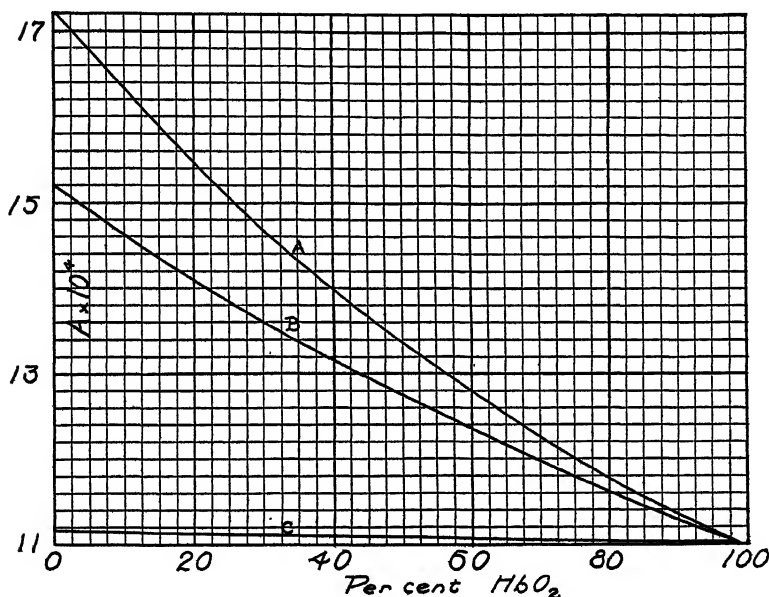


FIG. 2. Changes in the value of the absorption ratio for mixtures ( $A_{\text{mix.}}$ ) of oxyhemoglobin with methemoglobin (Curve A), reduced hemoglobin (Curve B), and carboxyhemoglobin (Curve C).

Again the ratio value indicates the purity, and total pigment is estimated in the usual manner.

### *Routine Application*

It may be well at this point to describe the routine examination of a sample of blood giving those points in technique which have been found advantageous. 1 cc. of blood is diluted to 100 or 200 cc. with 0.4 per cent ammonia or 1 per cent sodium carbonate. Read-

ings can be made most efficiently when the transmission lies between 10 and 25 per cent and one soon learns to estimate the necessary dilution to give readings within this range. Two essentials must be observed at this point: first, that the solution be well mixed and oxygenated; secondly, that the solutions are crystal-clear since any turbidity is a source of error. We have found the Manufacturing Chemists Association type of volumetric flask particularly useful for dilutions where oxygenation must be carried on simultaneously.

Whenever possible, readings should be made on a 1 cm. tube in order to simplify calculations. Transmission is read at the usual two wave-lengths. We have found five readings at each wave-length for each sample, checked by a similar set of readings on a second sample preferably from a new dilution when possible, to give the most accurate results. The average value of the transmission at each wave-length ( $T_\lambda$ ) is then used in the calculations as indicated above.

If a solution of oxyhemoglobin and methemoglobin diluted 1:100 and in a 1 cm. tube be taken as a specific example, the average value of  $T_{540}$  is found to be 0.138 and  $T_{560}$  to be 0.238, the reciprocals of which are 7.24 and 4.20 respectively. Finding the logarithms of these we have 0.8597 and 0.6232. Using these figures in Equation 10 or 11, we obtain

$$R = \frac{-\log T_{540}}{-\log T_{560}} = \frac{0.8597}{0.6232} = 1.392$$

From Fig. 1 we find that a ratio value of 1.392 is equivalent to 39 per cent oxyhemoglobin and 61 per cent methemoglobin. Fig. 2 shows that the absorption ratio of such a mixture ( $A_{\text{mix.}}$ ) is 0.00141. Therefore, the total pigment  $c$  in the dilution will be by Equation 5

$$c = \frac{A_{\text{mix.}}}{\dots} \cdot -\log T$$

or in gm. per cent of the original

$$\frac{0.8597 \cdot 0.00141}{1} \quad \text{dilution} \cdot 100$$

$$c = 0.001212 \cdot 100 \cdot 100$$

$$c = 12.12 \text{ gm. per cent total pigment}$$

Hence, we have 4.74 gm. per cent oxyhemoglobin and 7.39 gm. per cent methemoglobin.

Usually, the nature of the second pigment is known or suspected from the history of the sample, but under certain conditions no information is available. Since the determination of the ratio value ( $R$ ) is not specific, one must turn to other means for the recognition of the unknown. There are certain spectrophotometric characteristics of the various pigments which assist materially in the qualitative differentiation. A consideration of the absorption curves given in Fig. 3 will at once make these points obvious. This graph shows the absorption curve of oxyhemoglobin (Curve B), carboxyhemoglobin (Curve A), and alkaline methemoglobin (Curve C). In an unknown solution we may at once dismiss the possibility of reduced hemoglobin as one of the pigments since the process of dilution and mixing is sufficient to oxygenate it. We are, therefore, concerned with only the more stable pigments. Of these the more common are carboxy- and methemoglobin. They may be recognized at once by certain definite changes in the absorption at wave-length  $575 m\mu$ .

If one finds a low ratio value but a density at  $575 m\mu$  equal or greater than that at  $540 m\mu$  it will be seen from Fig. 1 that the second pigment is undoubtedly carboxyhemoglobin. If, on the other hand, the density is lower at  $575 m\mu$  than it is at  $540 m\mu$ , then the second pigment is methemoglobin, for it is obvious from the graphs that the absorption at  $540 m\mu$  is much greater than that at  $575 m\mu$  in the case of methemoglobin. It is also obvious that it is unsafe for one to hold too rigidly to these criteria unless the observer is certain no other pigments than those mentioned above are present. In cases of unknown pigments the whole absorption curve must be plotted and characteristics of each type of blood pigment be considered. Such cases are, however, rare. The reader is referred to Haurowitz (14) for an extensive chart of absorption curves of various hemoglobin combinations. Even so the data found may be misleading and it is only by examination of known solutions containing the suspected mixtures that information may be obtained.

It is of interest to compare the results of analyses made by the procedure outlined above with certain standard methods. The results of various analyses are given in Table IV. In Table IV



we have compared the results found by the spectrophotometer given in the left-hand group with those found by some second method given on the right. The first series are on oxyhemoglobin,

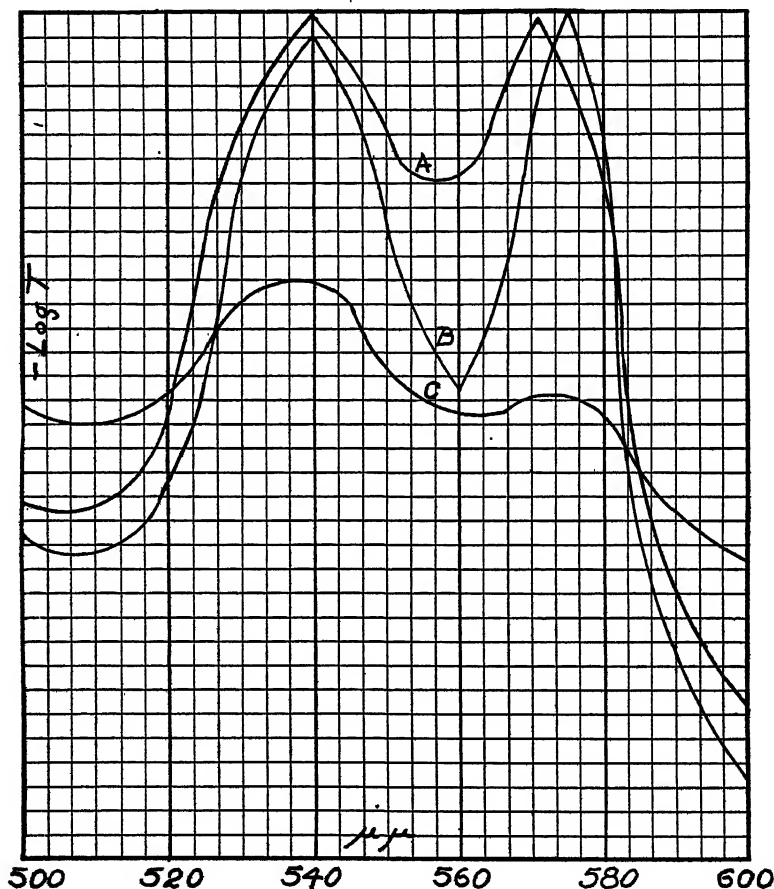


FIG. 3. The variation of optical density ( $-\log T$ ) with wave-length for carboxyhemoglobin (Curve A), oxyhemoglobin (Curve B), and methemoglobin (Curve C).

four of which are compared with determinations made on the Van Slyke apparatus. The fifth is on a dilute sample of hemoglobin where the second method is the colorimetric benzidine method

described by Bing and Baker (16). The agreement between the results is close and within the limits of error of either piece of apparatus. The same is true of the next series of readings given, namely those on the analysis of carboxyhemoglobin. The first figures are a comparison with the gasometric method and the others

TABLE IV

*Comparison of Results Found by Spectrophotometric Method with Those by Other Methods*

Pigments	Concentration by spectrophotometer			Concentration by second method			Remarks (second method)
	HbO <sub>2</sub>	Second pigment	Total pigment	HbO <sub>2</sub>	Second pigment	Total pigment	
	gm. per cent	per cent	gm. per cent	gm. per cent	per cent	gm. per cent	
HbO <sub>2</sub>	8.21			8.39			Gasometric
	11.99			11.96			"
	8.15			8.07			"
	0.18			0.172			Colorimetric (16)
HbCO	14.01			13.87			Gasometric
			2.58			2.42	Colorimetric (16)
			3.01			2.83	
			2.09			2.10	
			2.62			2.32	
	per cent			per cent			
HbO <sub>2</sub> + HbCO	38	62		40	60		Colorimetric (17)
	25	75		35	65		" (17)
	41	59		40	60		" (17)
Hb + MHb	42	58	10.14			10.02	Gasometric
	54.5	45.5	9.91			10.02	"
	74.5	25.5	10.01			10.02	"
	83	17	10.04			10.02	"
	91.5	8.5	9.96			10.02	"

with the benzidine method. Again the deviation is small. Although owing to the low concentration the variation may appear large, the same is true in regard to any method under the same conditions. The next group is on mixtures of oxy- and carboxy-hemoglobin in postmortem blood from a suspected case of CO poisoning. Three samples of blood were taken from various parts

of the body after embalming. The results found by the spectrophotometer are compared with estimations made by the colorimetric method of Sayers and Yant (17). Even under the unfavorable conditions we find close approximations.

The final series represents a series of determinations made on a blood of known total pigment concentration which was divided and one portion partially converted to methemoglobin with potassium ferricyanide and then the two mixed. The ratio value was then determined and the total pigment computed. As may be seen the results are within 1 per cent of the average (10.01 gm. per cent).

While these scattered readings by no means present a complete picture of the possibilities of the method, they do show beyond question its adaptability and the fact that it is comparable in accuracy with other methods in common use.

#### SUMMARY

A revision of the earlier methods of quantitative spectrophotometry of the blood pigments is presented.

By means of previously prepared graphs, the data for which are given, it is possible to analyze a sample of blood for total pigment, oxygen content and capacity, and concentration of a second pigment (carboxyhemoglobin, methemoglobin, or reduced hemoglobin). The examination may be made on a small sample of blood, 0.05 cc., and in a very short time, with a high degree of precision.

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# A CARBOHYDRATE BALANCE SHEET FOR THE ADRENALINIZED FASTING RAT SACRIFICED IN THE RECOVERY PERIOD

By M. LOUISA LONG AND FRITZ BISCHOFF

*(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara)*

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In striking a carbohydrate balance for the fasting adrenalinized rabbit (1), 85 per cent of the carbohydrate was accounted for by analysis for muscle and liver glycogen, muscle sugar, and urine sugar, but only 60 per cent when total carbohydrate (fermentable reducing power of acid hydrolysate) determinations were used in the calculations instead of muscle sugar and glycogen. Cori (2) had previously accounted for 85 per cent of the carbohydrate in the adrenalinized rat by determining the changes in liver and body glycogen and blood sugar. In view of the difference in results obtained when total carbohydrate was substituted for glycogen and muscle sugar in our rabbit experiments, it appeared important to extend the total carbohydrate studies to the rat.

## EXPERIMENTAL

The rats were divided into three groups, one set serving as controls, one set receiving 0.02 mg. of adrenalin per 100 gm. subcutaneously, and one set receiving 0.10 mg. of adrenalin per 100 gm. subcutaneously. The lower dose of adrenalin is not glycosuric nor does it lower the muscle sugar. The higher dose, which is sublethal, produces both of these phenomena. All animals were fasted 24 hours. The low dose of adrenalin was given the 20th hour, the high dose the 16th hour of fasting. The animals were sacrificed the 24th hour by spinal transection according to the method of Anderson and Macleod (3), the time of dosage being so arranged that the adrenalinized animals were in the recovery period at the time of sacrifice. Total fermentable

carbohydrate was determined for the hind legs, for the liver, and for the other internal organs according to the method previously described (1). Total non-fermentable carbohydrate was estimated semiquantitatively by the modified Molisch test of Foulger (4). Urine sugar and urine and muscle lactic acid were also determined, the latter procedures by the method of Mendel and Goldscheider (5), preceded by an ether extraction of lactic acid according to Clausen (6).

It was not feasible to make all the determinations for the same animal. Not less than six determinations were made for each substance to be analyzed. The same animals served for the three sets of total carbohydrate determinations. Another set was used for urine sugar and lactic acid determinations and a third set for tissue lactic acid determinations. The urine was collected for a 24 hour period, 8 hours after adrenalin dosage. The tissues and their filtrates for tissue lactic acid determination were subjected to exactly the same procedure as that used for total carbohydrate determination. Any carbohydrate that might reach the lactic acid stage instead of the glucose stage during the hydrolysis in the total carbohydrate determination would therefore be included in the tissue lactic acid determination.

In all determinations of fermentable total carbohydrate, glucose was added to the filtrate after fermentation and the recovery determined. For the muscle filtrates the mean recovery was 96 per cent, a figure which agrees exactly with that found for filtrate of rabbit muscle. For the organs, exclusive of the liver, recovery was quantitative, an observation which was not expected since the fermentable carbohydrate represents only one-sixth of the reducing power of such filtrates.

### *Results*

*Non-Fermentable Carbohydrate*—In estimating the non-fermentable carbohydrate by Foulger's modification of the Molisch test, glucose solutions were used as standards. The values so obtained obviously can have no absolute significance, since it is definitely known that the non-fermentable carbohydrate is not glucose, since the color, particularly for the filtrates prepared from the organs, is less violet than that given by glucose, and since the effect of other substances is not known. A comparison of the

values for control and for adrenalinized animals should, however, reflect changes that might be induced. The results obtained were remarkably uniform and indicated that adrenalin had no significant effect upon the non-fermentable carbohydrate. Per 100 gm. of rat, the figures for the muscle non-fermentable carbohydrate<sup>1</sup> were  $12 \pm 1.4$  for the controls,  $16 \pm 4.0$  for the rats which had received 0.02 mg. of adrenalin, and  $12 \pm 1.4$  for the rats which had received 0.10 mg. of adrenalin. Per 100 gm. of rat, the values for the non-fermentable carbohydrate of the liver were 3.2, 3.2, and 3.0 mg., respectively, for the three groups of animals. Per 100 gm. of rat, the values for the non-fermentable carbohydrate of the organs were  $26 \pm 3.0$ ,  $23 \pm 4.0$ , and  $24 \pm 5.0$  for the three groups, respectively. Per 100 gm. of substance analyzed, the non-fermentable carbohydrate was highest in the organs and lowest in the muscle.

*Urine and Tissue Lactic Acid*—The experience of other workers (7) indicates that the amount of lactic acid lost through the kidneys during adrenalin activity is not appreciable. Since a sublethal dose of adrenalin was given in one series of the present experiments, it was necessary to establish whether lactic acid excretion by the kidneys was appreciably increased during the activity of this high dosage. Urine samples of six control and twelve adrenalinized rats were collected for a 24 hour period, the dose of adrenalin being administered 8 hours after collection was begun. The results showed that the lactic acid excretion was doubled or tripled, but the amount, 0.4 mg. per 100 gm. of rat, is less than 0.5 per cent of the total carbohydrate of the rat, so may be omitted in the carbohydrate balance.

There was no significant difference in muscle lactic acid content between control and adrenalinized rats (0.1 mg. of adrenalin per 100 gm. of rat).

Control values, mg. per 100 gm., 150, 128, 52, 135, 147, 69; mean,  $114 \pm 18$ .

Values of adrenalinized rats, mg. per 100 gm., 89, 98, 153, 75, 83; mean,  $99 \pm 14$ .

*Urine Sugar*—Urine sugar was determined for ten rats which had received 0.1 mg. of adrenalin per 100 gm. The rats were

<sup>1</sup> The estimation of pentoses by the orcinol Bial reagent gives much higher values (4 times).



placed in metabolism cages in groups of two. The individual values, sugar excreted per 100 gm. of rat, were 31, 24, 36, 55, and 46 mg. What effect the grouping has upon the calculation of the degree of variation cannot be estimated. In calculating the standard deviation of the mean, five was taken as the number of samples.

TABLE I

*Total Fermentable Carbohydrate Content of Leg Muscle, Liver, and Internal Organs Other Than Liver of Fasting Control and Adrenalinized Rats*

	Weight of			Total fermentable carbohydrate		
	Rat	Liver	Organs*	Muscle	Liver	Organs*
	gm.	gm.	gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.
Controls fasted 24 hrs.	210	6.5	19		580	
	228	7.0	25.5	305	319	184
	200	6.2	23	406	182	204
	166	5.5	20.8	295	295	242
	310	8.8	31.5	400	946	161
	340	10.2	29.8	389	1050	124
	220	6.3	26.8	365	732	190
0.02 mg. per 100 gm. adrenalin. Killed 4 hrs. later	335	7.0	18.2		1395	
	193	5.8	21.2	258	1415	126
	160	5.5	18.0	234	829	234
	265	8.5	22.6	140	1670	208
	250	9.2	16.4	235	382	367
	230	7.2	17.6	187	2450	255
	200	5.8	22.1	209	928	354
0.1 mg. per 100 gm. adrenalin. Killed 8 hrs. later	335	9.2	24.3	168	1255	185
	230	5.0	16.0	167	327	216
	225	7.0	20.0	150	1015	292
	182	5.6	18.0	135	405	239
	172	5.5	17.7	210	199	220
	185	6.7	21.1	178	559	204

\* Organs other than liver.

*Total Fermentable Carbohydrate*—The analytical values for total fermentable carbohydrate per 100 gm. of substance are given in Table I. The measurement of variability has not been included for these figures. Calculated on the basis of mg. per 100 gm. of rat, it may be found in the balance sheet (Table II).

*Balance Sheet*

The calculations given in the balance sheet are based on the assumption that the peripheral tissue constitutes 50 per cent of the body weight and that carbohydrate is the source of lactic acid. The results show no significant changes in the tissue lactic acid values or internal organ (other than liver) total fermentable carbohydrate values. The total fermentable carbohydrate content of the muscle is considerably reduced following the action of

TABLE II

*Carbohydrate Balance Sheet of the 100 Gm. Fasting Rat 4 Hours after Subcutaneous Injection of 0.02 Mg. of Adrenalin per 100 Gm. and 8 Hours after Subcutaneous Injection of 0.1 Mg. of Adrenalin per 100 Gm.*

	Control	0.02 mg. adrenalin per 100 gm.	0.10 mg. adrenalin per 100 gm.
	mg.	mg.	mg.
Blood sugar.....	10	10	10
Muscle total carbohydrate (fermentable).....	181 $\pm$ 10	106 $\pm$ 9	84 $\pm$ 6
Muscle lactic acid.....	57 $\pm$ 9	(57 $\pm$ 9)	49 $\pm$ 7
Liver total carbohydrate (fermentable).....	17 $\pm$ 4	39 $\pm$ 8	19 $\pm$ 5
Organ* total carbohydrate (fermentable).....	21 $\pm$ 3	23 $\pm$ 4	20 $\pm$ 2
Urine sugar.....			38 $\pm$ 5
	286 $\pm$ 15	235 $\pm$ 16 82 per cent	220 $\pm$ 14 76 per cent

The figures in parentheses were arrived at by using values obtained for controls.

\* Organs other than liver.

adrenalin, but the changes are not nearly as marked as those observed in earlier work on the rabbit. The lower dose of adrenalin brought about a marked rise in the total fermentable carbohydrate of the liver, an observation which was to be expected on the basis of previous work on the glycogen changes under the same conditions. The sublethal dose of adrenalin, however, brought about no change in the carbohydrate content of the liver.

The balance sheet shows that the rat adrenalinized with 0.02 mg. of adrenalin contains 82 per cent, the rat adrenalinized with

0.1 mg. of adrenalin contains 76 per cent, of the fermentable carbohydrate or its equivalent of lactic acid found in the control rat. The absolute differences in carbohydrate, 51 and 66 mg. respectively, would appear to be significant, since they approach 3 times the standard deviation of the mean. It should be noted that two-thirds of the calorogenic effect of the lower dosage of adrenalin could be accounted for by the oxidation of 51 mg. of carbohydrate.<sup>2</sup>

Only 30 per cent of the carbohydrate lost from the peripheral tissues is accounted for by an increase in the hepatic carbohydrate content in the case of the rat which had received 0.02 mg. of adrenalin per 100 gm., while 41 per cent is accounted for by liver and urine carbohydrate increases for the rat which had received 0.1 mg. of adrenalin per 100 gm. In spite of the rather large individual variation, the difference between the carbohydrate lost from the peripheral tissues and that gained by the liver or excreted in the urine is 3.3 and 4.5 times, respectively, the joint standard deviation of the mean (square root of the sum of the squares of all means used), and would therefore appear significant. These figures are in disagreement with the work (2, 7) based on glycogen analysis. The difference in results may be traced to three sources: (1) a greater difference in muscle fermentable carbohydrate changes than in muscle glycogen changes following adrenalin, (2) a lesser difference in hepatic fermentable carbohydrate changes than in glycogen changes following adrenalin, and (3) elimination of the error introduced by calculating tissue sugar changes.

#### SUMMARY

Carbohydrate balances for fasting rats in the recovery period following the subcutaneous administration of 0.02 or 0.10 mg. of adrenalin per 100 gm. of rat were struck by determining for suitable series of controls and adrenalinized animals the total fermentable and non-fermentable carbohydrate of legs, liver, and other organs, the muscle and urine lactic acid, and the urine sugar. Only 30 to 40 per cent of the carbohydrate lost from the peripheral

<sup>2</sup> Based on Cori's data that 0.02 mg. of adrenalin per 100 gm. of rat increases the metabolism 13 per cent over a 4 hour period. Per 100 gm. of rat, the increase is equivalent to 0.3 calories.

tissues is accounted for by a gain in liver carbohydrate or excretion of urine sugar.

Of the total fermentable carbohydrate of the whole body of the control rat, 82 per cent was accounted for in the rat adrenalinized with 0.02 mg. of adrenalin per 100 gm., 76 per cent for the rat adrenalinized with 0.1 mg. of adrenalin per 100 gm.

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## STUDIES ON CRYSTALLINE INSULIN\*

### XVI. THE ACTION OF AMMONIUM HYDROXIDE AND OF IODINE ON INSULIN

By H. JENSEN, E. SCHOCK, AND E. SOLLERS

(From the Department of Pharmacology, the Johns Hopkins University,  
Baltimore)

(Received for publication, July 13, 1932)

In a previous communication the methods which may be employed in the elucidation of the chemical constitution of insulin have been outlined (1). In this paper we shall confine ourselves to a brief report of our observations on the action of ammonium hydroxide and of iodine on insulin.

#### *Action of Ammonium Hydroxide on Insulin*

Witzemann and Livshis (2) claim that insulin may be reversibly inactivated by treatment with approximately 0.5 N ammonium hydroxide for several days. They suggest a tautomeric rearrangement as the chemical basis of this phenomenon. We have studied this reaction in detail, carrying out a large number of experiments at various concentrations of ammonium hydroxide. As we shall show later, we found it difficult to duplicate our results in several instances. We shall confine the discussion temporarily, therefore, to the more important experiments.

Subcutaneous injection of insulin which has been dissolved in 0.5 N ammonium hydroxide immediately prior to injection seems to indicate a partial inactivation of the hormone. Intravenous injection of similarly treated material, however, shows no loss in hypoglycemic activity (see Experiments 1 and 2, Table I). If the ammoniacal solution is diluted with water in the proportion of 1:15, subcutaneous injection shows undiminished activity (see Experiment 3, Table I). It seems probable that the alkalinity

\* An investigation carried out under grants from the Carnegie Corporation of New York and Eli Lilly and Company, Indianapolis.

TABLE I  
*Action of Ammonium Hydroxide on Insulin*

Experiment No.	Crystalline insulin	No. of rabbits	Average weight	Dose per animal	Average blood sugar, mg. per 100 cc.							Convulsions
					Normal	15 min.	30 min.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	
		kg.	mg.									
1	Dissolved in 0.5 N NH <sub>4</sub> OH; just before subcutaneous injection	71.94	$\frac{1}{30}$	119					98		88	0
		81.98	$\frac{1}{20}$	118					109		106	
2	Same; intravenous	122.07	$\frac{1}{30}$	119	86	70	59					5
3	Same; diluted 1:15 before subcutaneous injection	121.98	$\frac{1}{30}$	123			62			71		6
4	Same; 6 days at room temperature. Subcutaneous	51.98	$\frac{1}{30}$	122			122		114			0
		72.08	$\frac{1}{20}$	112					88			0
5	Same; 6 days at room temperature. Intravenous	101.97	$\frac{1}{30}$	119	104	95	106					0
		31.98	$\frac{1}{2}$	103			59			80		0
6	Same; 6 days at room temperature. Acidified 3 hrs. before intravenous injection	101.98	$\frac{1}{30}$	127	108	91	99					0
7	Dissolved in N NH <sub>4</sub> OH; just before subcutaneous injection	31.95	$\frac{1}{30}$	119					88		89	1
		172.08	$\frac{1}{20}$	112					101		100	0
8	Same; intravenous	172.06	$\frac{1}{20}$	122			52			83		7
9	Same; 6 days at room temperature. Subcutaneous	41.77	$\frac{1}{20}$	116					108			0
		32.25	$\frac{1}{15}$	134					119			0
		42.08	$\frac{1}{10}$	127					108		106	0
		42.10	$\frac{1}{8}$	118					118		119	0
		42.03	$\frac{1}{8}$	128					130		103	0
10	Same; 6 days at room temperature. Intravenous	72.21	$\frac{1}{20}$	118			106			114		0
		52.15	$\frac{1}{15}$	115	108		85			87		0
11	Same; 6 days at room temperature. Acidified night before intravenous injection	132.05	$\frac{1}{2}$	124		80	95			130		0
12	Treated with 2 M alcoholic NH <sub>4</sub> OH 5 days. Dissolved in 0.01 N NH <sub>4</sub> OH just before subcutaneous injection	82.10	$\frac{1}{20}$	110					81		94	2
		81.85	$\frac{1}{10}$	123					98		105	5

of the solvent affects the tissues so as to prevent, or at least diminish absorption. This explains the apparent inactivation observed in subcutaneous injections of 0.5 N ammonia solutions.

Treatment of insulin with 0.5 N ammonium hydroxide for a period of 6 days at room temperature (approximately 22°) partially inactivates the hormone. The material so treated shows loss of activity on both subcutaneous and intravenous injection (see Experiments 4 and 5, Table I). In contradiction to the observations of Witzemann and Livshis, however, we were not able to reactivate this material by acid treatment (see Experiment 6, Table I). Using N ammonium hydroxide under the same conditions, we have obtained similar and even more striking results (see Experiments 7 to 11, Table I). After treating insulin with weaker concentrations of ammonia for several days at room temperature a slight inactivation is observed on intravenous injection. Subcutaneous injection of insulin immediately after solution in these weak concentrations of ammonia shows varying results: in some cases the full expected activity is obtained; in others, inhibition of the absorption of the insulin is observed. Due to the inconsistency of these results, the experimental data are omitted for the sake of brevity. With still weaker concentrations of ammonia (below 0.1 N), no inhibiting effect is observed.

These findings are in perfect agreement with unpublished experiments, pertaining to the same problem, conducted by Dr. O. Wintersteiner in this laboratory in 1929. In addition to the results recorded above, it has been found that a freshly prepared solution of crystalline insulin in 1 N ammonia, which was inactive as such on subcutaneous injection, showed the full expected strength after acidification.

On the basis of these facts we are inclined to interpret the results of Witzemann and Livshis as due to the decreased absorption of the ammoniacal solutions used because of their action on the animal tissues. Acidification of these solutions and the ability of the tissues to absorb the weakly acid medium without difficulty would explain the apparent acid reactivation observed by these workers.

It may be mentioned that treatment of insulin with 2 M alcoholic ammonium hydroxide for 5 days at room temperature partly



inactivates the hormone (Experiment 12, Table I). Attempts to reactivate this material by acid have thus far failed. The chemical changes in the inactivation of insulin by ammonium hydroxide are probably similar to those observed under the influence of sodium hydroxide (3).

Freudenberg and his coworkers (4) have also studied the action of ammonia on insulin. They found that insulin is completely inactivated by 1 N ammonia at 34° in the course of 8 hours. No details as to the preparation of the solutions and the mode of injection are given.

Both 0.5 N and N ammonium hydroxide solutions were found to have no action on the blood sugar.

#### *Action of Iodine on Insulin*

Brand and Sandberg (5) have suggested a possible iodometric estimation of insulin. They claim that if the insulin preparation is of sufficient purity the unitage of the material may be determined iodometrically. Repeating this work with various insulin preparations, including crystalline insulin, we find, in agreement with these investigators, that the amount of iodine absorbed after 17 hours is approximately proportional to the activity of the insulin preparation used. The amount of iodine absorbed immediately, however, shows no such relationship. The use of an iodometric titration of insulin is dependent upon the non-reaction of the impurities present in the assayed material. Since the groupings acted upon by iodine under these conditions are by no means confined solely to the insulin molecule, we doubt that it is feasible to use the iodometric titration as a basis for the determination of the physiological strength of insulin preparations.

Blatherwick and his coworkers (6) have also studied the action of iodine on a number of amorphous insulin preparations. These workers find that insulin is inactivated by a definite amount of iodine. We have investigated this reaction in an effort to ascertain which of the groups affected are concerned in the physiological action of the hormone.

We have found that the addition of 0.3 cc. of about 0.01 N iodine to 1 mg. of crystalline insulin completely and immediately inactivates the material. When less iodine is used, partial inactivation is obtained. Comparison of the data in Tables II

and III shows that the amount of iodine necessary for complete inactivation (0.3 cc. of 0.01 N iodine per mg. of crystalline insulin) is in close agreement with the amount of iodine (0.294 cc. of 0.01 N iodine per mg. of crystalline insulin) immediately taken up by the insulin.

TABLE II  
*Absorption of Iodine by Insulin*

Material	Amounts	0.01 N iodine absorbed immediately	0.01 N iodine absorbed after 17 hrs.
	mg.	cc.	cc.
Lilly's insulin, 17 to 18 units	1	0.266	0.861
	2	0.443	1.691
	3	0.684	2.324
Squibb's insulin, 20 units	1	0.383	1.093
	2	0.709	2.039
	3	0.967	2.865
Crystalline insulin, 24 units	1	0.294	1.209
	2	0.451	2.203
	3	0.592	3.115

TABLE III  
*Inactivation of Insulin by Iodine*

0.01 N iodine per mg.	No. of rabbits	Average weight	Dose per animal	Blood sugar		Convulsions
				Normal	1½ hrs.	
cc.		kg.	mg.	mg. per 100 cc.	mg. per 100 cc.	
0.3	6	2.00	$\frac{1}{2}$	137.5	101	0
0.2	6	2.15	$\frac{1}{2}$	136	77	2
0.15	6	1.925	$\frac{1}{10}$	128.5	65	2
0.10	12	1.99	$\frac{1}{10}$	138	65.6	4

It is known that under the conditions employed iodine will react with various amino acids: cystine, tyrosine, and histidine. These amino acids have been previously isolated from the insulin molecule. We have found that tyrosine, histidine, cystine, and certain cystine derivatives absorb definite amounts of iodine within a few seconds after addition of an iodine solution. Under

the influence of iodine the disulfide linkage of cystine is oxidized, the final product being cysteic acid. We are inclined to assume that the inactivation of insulin under the conditions employed in this work is due mainly to the oxidation of the disulfide linkages in the molecule. The possibility must be considered, however, that the iodine may also react with the apparently physiologically important free amino groups (1).

The addition of acid to the reaction mixture after the action of iodine on insulin has been completed precipitates an amorphous material which is acid in nature, insoluble in water and organic solvents, soluble in alkali, and reprecipitated by acid. This is probably a split-product of insulin, possibly a cysteic acid peptide, formed as the result of the oxidation of the disulfide linkage to the sulfonic acid group. We are at present engaged in studying the composition of this product in the hope of ascertaining which amino acid is linked to cystine in the insulin molecule. The possible importance of certain cystine peptides in the physiological action of insulin has already been suggested in a previous communication (1).

#### EXPERIMENTAL

The treatment of insulin with ammonium hydroxide was carried out in small glass bottles with ground-in stoppers in order to prevent loss of ammonia by vaporization.

In the iodine experiments 0.01 N iodine and 0.005 N thiosulfate solutions were used. These reactions were also carried out in glass-stoppered bottles. From 1 to 3 mg. of insulin, 3 cc. of phosphate buffer, 5 cc. of 0.01 N iodine solution (final pH about 7.6) were titrated, either immediately, or after standing 17 hours at room temperature (about 22°) with the thiosulfate after the solution was made 0.1 N acid with hydrochloric acid. A blank was always run simultaneously and the value of the iodine was taken from this figure.

In the inactivation experiments the insulin was dissolved in M/15 Na<sub>2</sub>HPO<sub>4</sub>, the iodine solution added, and the material injected immediately.

The data given in Tables I to III represent averages of a number of experiments.

## SUMMARY

The action of 0.5 N and of N ammonium hydroxide solutions on crystalline insulin has been studied. It is suggested that ammonia solutions of these concentrations prevent the absorption of insulin when given subcutaneously. On standing for 6 days at room temperature in ammonia solutions of these concentrations, insulin is partially inactivated. In contradiction to Witzemann and Livshis (2) we have found that this insulin cannot be re-activated by the addition of acid. A possible explanation is given for the results of Witzemann and Livshis.

In studying the action of iodine on insulin, it has been found that a definite amount of iodine will inactivate insulin immediately. This quantity was found to be much smaller than that absorbed in the course of 17 hours. We assume that the inactivation is due mainly to the oxidation of the disulfide linkage of insulin.

The data presented here are to be interpreted from a qualitative rather than a quantitative point of view.

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## THE ESTRUAL CYCLE IN RATS ON A MANGANESE-FREE DIET

BY ELSA R. ORENT\* AND E. V. MCCOLLUM

(From the Biochemical Laboratory, School of Hygiene and Public Health,  
the Johns Hopkins University, Baltimore)

(Received for publication, July 19, 1932)

In a previous publication (1) it was shown that female rats fed a diet free from manganese grow to maturity in an apparently normal manner and go through normal estrual cycles.

Other investigators (2) found, at about the same time, that mice and rats reared on a diet of cow's whole milk, which is low in manganese, supplemented with iron and copper, failed to ovulate normally. Animals reared on the same diet with the addition of manganese or iodine, or both, exhibit normal estrual cycles.

In view of these discrepant observations, the experiments reported in the present paper were undertaken. Four different experiments were carried out. Rats from our stock colony were taken at weaning and placed in cages with screen bottoms. Each group consisted of five female rats. Group I was fed the manganese-free diet. Group II received the manganese-free diet with the addition of 0.005 per cent manganese. Group III was given a diet composed of cow's certified whole milk containing 0.5 mg. of copper and 1.0 mg. of iron per 100 cc. of milk. Group IV received the diet given to Group III supplemented with 0.5 mg. of manganese per 100 cc. of milk. The milk diets were fed *ad libitum*.

The technique for preparing the manganese-free diet, as well as the general laboratory technique, has been described previously (1).

All the animals were given distilled water daily and iodine water once a week.

The ferric chloride, copper sulfate, and manganese chloride used in the experiments were high grade salts (Baker's Analyzed).

\* National Research Fellow in Biological Sciences (Biochemistry) 1930-32.

The estrual rhythm of these rats was studied, as previously described, by taking vaginal smears daily according to the method used by Long and Evans (3). The vaginal examination was started when the rats had been on the diets for 6 weeks, at an age of about 67 days. These animals were observed for a period of 150 days. In all of the animals studied the frequency of the estrual cycle appears to be normal, namely 4 to 6 days.

Waddell, Steenbock, and Hart (2) have observed that on milk diets growth was below normal. Good weights and normal maintenance are attained, but neither the increases in weight nor the final weights were optimum. They suggest "that this may be due to a low intake of calories because of the large amount of fluid that has to be ingested and that a limiting factor may be the capacity of the animal to handle sufficient quantities."

Kemmerer, Elvehjem, and Hart (4) have shown that addition of manganese to a diet of cow's whole milk supplemented with iron and copper has a measurable effect upon the growth.

Our present studies show that the growth curves of the rats fed the manganese-free diet do not differ from those receiving manganese, which observations confirm our previous experiments. The growth of the rats on the milk diets was not as good, although fair weights were obtained.

#### SUMMARY

1. A study has been made of the reproductive and growth performances of rats on the manganese-free diet, the same diet supplemented with 0.005 per cent manganese, on a diet of cow's certified whole milk containing 0.5 mg. of copper and 1.0 mg. of iron per 100 cc. of milk, and the above milk diet supplemented with 0.5 mg. of manganese per 100 cc. of milk.

2. All the animals studied exhibited normal estrual cycles.

3. Rats on the manganese-free diet and those on the diet supplemented with manganese grew in an apparently normal manner.

The rats on the milk diets showed somewhat inferior growth.

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## FURTHER STUDIES ON THE WAX-LIKE COATING OF APPLES

By K. S. MARKLEY, STERLING B. HENDRICKS, AND  
CHARLES E. SANDO

*(From the Bureau of Plant Industry and the Bureau of Chemistry and Soils,  
United States Department of Agriculture, Washington)*

(Received for publication, July 6, 1932)

### INTRODUCTION

Sando (1), in 1923, examined the petroleum ether extract of Ben Davis apple peels, from which he isolated a paraffin hydrocarbon (m.p. 63.5–64°) and a secondary alcohol (m.p. 81–81.5°). The hydrocarbon was identical with that previously isolated from Ben Davis apple peels by Power and Chesnut (2) (1920) who reported for it a melting point of 63° and assumed it to be triacontane,  $C_{30}H_{62}$ . Elementary analysis and the melting point of the secondary alcohol and its acetate corresponded with 14-heptacosanol prepared by Kipping (3) and it was therefore assumed to be identical with this alcohol.

Channon and Chibnall (4) (1929) reported the isolation of *n*-nonacosane and di-*n*-tetradecyl ketone from cabbage leaves and intimated that the hydrocarbon isolated by Sando may have been *n*-nonacosane contaminated by the corresponding or a similar ketone. The investigators mentioned above identified their products with the aid of x-ray diffraction from the plane of greatest spacing. This method, which was not available to Sando at the time he attempted to identify the hydrocarbon from apple peels, affords a most trustworthy means for determining the number of carbon atoms in such compounds.

For several years two of the authors have been engaged upon the problem of isolation and utilization of the constituents of the wax-like coating of apples and have during that time obtained 25 to 30 pounds of crude hydrocarbon fraction from such materials as apple pomace, peels, and cuticle. Following the publication of



Channon and Chibnall's paper we undertook a reinvestigation of the hydrocarbon fraction of apple cuticle directed particularly toward the possible isolation of a ketone. Coincident with the completion of this work but before final incorporation in manuscript form, Chibnall and his associates (5) reported a very thorough examination of the petroleum ether extract of apple peels, which paralleled our recent work on apple cuticle and led to substantially the same results.

### *Material and Methods*

In view of the very detailed description of the methods of Chibnall *et al.*, which in many respects were similar to those followed by us, we shall confine our report to a brief description of the material investigated, methods applied, and evidence for our present conclusions.

A quantity of air-dried Rome Beauty apple cuticle weighing 285 gm. and obtained by quartering the fruit, immersing it in dilute hydrochloric acid for 24 to 48 hours, stripping the cuticle with tweezers, washing with water, and drying on open trays was exhaustively extracted with hot petroleum ether. The extract, which was almost white and weighed 86 gm., was saponified with 3 per cent alcoholic potash, the alcohol partially removed by evaporation, and the saponification mixture poured into cold water. The emulsion thus formed was broken by the addition of common salt and the precipitate removed by filtration, dried, and extracted with successive portions of petroleum ether.

After evaporation of the petroleum ether, the residual material was heated with phthalic anhydride at 140° for 3 hours, the mixture cooled, and heated with alcohol to remove excess of reagent. The mixture was then poured into a dilute solution of sodium carbonate, whereupon a separation occurred. The filtered residue was shaken with more sodium carbonate solution and extracted with ether and the extract evaporated to dryness. The residue, amounting to 16 gm. and melting at 64.4–64.9°, was refluxed 3½ hours with hydroxylamine hydrochloride and sodium acetate dissolved in 200 cc. of 95 per cent ethyl alcohol to which 10 cc. of water had been added; after which the reaction mixture was diluted with a large volume of boiling water. On cooling, a white cake formed on the surface of the liquid which was washed thoroughly,

then dried, and weighed. Recovery was quantitative and the product appeared to consist wholly of hydrocarbon.

The dried cake was dissolved in hot petroleum ether (b.p. 35–40°), filtered, and an equal quantity of hot acetone added, filtered again, and the solvent allowed to evaporate spontaneously. After a time, star-shaped clusters of needles separated from the solution. The crystals were removed by filtration, washed with a mixture of petroleum ether and acetone, and then with acetone. Four additional fractions were obtained by further partial evaporation of the mother liquors. Each fraction was recrystallized from petroleum ether and acetone as before. Fractions 4 and 5 were combined and also treated with acetic anhydride for 3 hours,



FIG. 1. x-Ray diffraction patterns of (A) *n*-nonacosane from Rome Beauty apples, (B) 10-nonacosanol from Ben Davis apples.

followed by crystallization from a mixture of petroleum ether and acetone. In this manner 10.3 gm. of hydrocarbon were obtained, but no evidence of the presence of a ketone could be found.

#### *x-Ray Examination*

Fractions 1, 2, and 3 were submitted to x-ray examination.  $\text{Cu K}_\alpha$  radiation was used with a 10 cm. crystal to plate distance. The crystals were sufficiently well formed to allow pressing against a copper strip as a mount. The value of  $d_{hkl}$  for the plane of greatest spacing was  $38.8 \pm 0.3 \text{ \AA.}$ , (0001) of  $\beta \text{ Al}_2\text{O}_3$ ,  $d = 22.48 \text{ \AA.}$ , being used for reference. The presence of twelve orders of reflection from this plane on the original photograph as shown in Fig. 1, A indicates that the compound is quite pure. The spacings, within our 0.7 per cent error, are the same as that of the A form of *n*-

nonacosane, as determined by Piper and coworkers (6). These fractions are therefore probably pure *n*-nonacosane.

Diffraction patterns were also obtained from the original hydrocarbon and alcohol isolated from Ben Davis apple peels by Sando in 1923. The hydrocarbon was found to be identical with the nonacosane, isolated from Rome Beauty apple cuticle. The alcohol showed a long spacing of  $38.8 \pm 0.3$  Å. with the third, sixth, and ninth orders of reflection missing as seen in Fig. 1, *B*, an indication that the hydroxyl group is attached to the 9th or 10th carbon atom. In view of the work of Chibnall and coworkers the alcohol may be considered as 10-nonacosanol.

#### *Melting Point Determinations*

Melting point determinations were made with an electrically heated apparatus (7) and Wheeler totally immersed thermometers standardized by the United States Bureau of Standards just prior to their use. The hydrocarbon fractions from Rome Beauty apple cuticle melted at  $65.1^\circ$  and set to a solid at  $64.1^\circ$ . Evidence of the occurrence of transitions was obtained in the neighborhood of  $61^\circ$  and sharply at  $64.3^\circ$  on heating and an apparent transition at  $62.7^\circ$  on cooling. The transitions and melting points reported above are averages of ten determinations by two different observers. The first transition on heating occurred approximately  $4^\circ$  below the melting point as compared with  $6^\circ$  reported by Chibnall *et al.* and was difficult to detect even with the aid of a good optical system. The second transition on cooling could not be observed with any degree of certainty.

A redetermination of the melting point of the hydrocarbon previously isolated from Ben Davis apple peels with the same method and apparatus as in the case of the hydrocarbon from Rome Beauty apples gave substantially the same results but about  $0.4^\circ$  lower. Although the method of taking the melting points was varied with respect to rate of heating, size of capillary, and amount of filling, the observed melting point deviated but slightly from that recorded above. Fractions of the same hydrocarbon obtained from the petroleum ether extract of dogwood bracts and flowers (unpublished results) likewise gave similar results. In none of these cases have we observed the melting of what we consider a pure sample of nonacosane to be as low as reported by

Chibnall and his coworkers. Whether the difference in melting point is due to a difference in the manner of taking the melting point or to an actual difference in purity of material we are unable to state.

#### CONCLUSIONS

A reinvestigation of the petroleum ether extract of the wax-like coating of apples directed particularly toward the isolation of a ketone led to the following conclusions:

The petroleum ether extract of apple cuticle does not contain a ketone in isolable quantities.

The principal hydrocarbon comprising the bulk of the petroleum ether extract is *n*-nonacosane, m.p. 65.1°, and not triacontane as previously reported.

The secondary alcohol is 10-nonacosanol and not 14-heptacosanol, although both alcohols have the same melting points, as do also their corresponding acetates.

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## SERINEPHOSPHORIC ACID OBTAINED ON HYDROLYSIS OF VITELLINIC ACID

BY FRITZ A. LIPMANN\* AND P. A. LEVENE

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

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The discovery in nature of many new organic compounds containing a phosphoric acid residue and the realization of their biological significance have caused renewed interest in the natural conjugated phosphoric acids that have been known for a long time but whose structure had not been known. Among the latter are the phosphoproteins. The best known representatives of these are casein and vitellin. From the latter Levene and Alsberg<sup>1</sup> obtained by treatment with ammonia water (12 per cent) for 2 hours a substance of constant composition containing 10 per cent of phosphorus and about 14 per cent of nitrogen, having the ratio P:N = 1:3. 26 years later Posternak and Posternak<sup>2</sup> prepared a substance of similar composition by tryptic digestion of vitellin. Undoubtedly these authors were dealing with the vitellinic acid of Levene and Alsberg. Posternak and Posternak subjected the substance to hydrolysis and found among the products of hydrolysis a higher proportion of serine than had hitherto been found in any other protein. The high yield of ammonia on acid hydrolysis and the isolation of pyruvic acid on alkaline hydrolysis, according to these authors, also was indicative of a high proportion of serine.<sup>3</sup> On the basis of the experience of Bergmann and his coworkers,<sup>4</sup> such results should be expected from a serine peptide. Later, the

\* Fellow of the Rockefeller Foundation.

<sup>1</sup> Levene, P. A., and Alsberg, C. L., *J. Biol. Chem.*, **2**, 127 (1906-07).

<sup>2</sup> Posternak, S., and Posternak, T., *Compt. rend. Acad.*, **184**, 909 (1927).

<sup>3</sup> Posternak, S., and Posternak, T., *Compt. rend. Acad.*, **187**, 313 (1928).

<sup>4</sup> Bergmann, M., Miekeley, A., Weinmann, S., and Kann, E., *Z. physiol. Chem.*, **143**, 108 (1925). Bergmann, M., Miekeley, A., and Kann, E., *Z. physiol. Chem.*, **146**, 247 (1925).

authors prepared a substance with a ratio of  $P:N = 1:1.3$  and even  $1:1$ . Analytical data on these substances were not presented.

Contemporaneously with Posternak and Posternak, Rimington<sup>5</sup> was conducting investigations on casein peptone and prepared a substance with 7 per cent phosphorus and 10 per cent nitrogen, the ratio of  $P:N$  being  $1:3$ . He reached the conclusion that the phosphoric acid residue in his peptone was bound to hydroxyglutamic and hydroxybutyric acids. His conclusion, however, was disputed by Posternak<sup>6</sup> who insisted that the phosphoric acid residue was bound to serine in the substance from casein as well.

The present investigation was undertaken with a view to a more detailed study of the structure of vitellinic acid than was presented by Posternak and Posternak. The contributions of these authors are undoubtedly of great importance but many of their results are of a qualitative character.

The vitellinic acid was prepared by the procedure of Levene and Alsberg with the sole modification that for purification it was precipitated from an aqueous solution by means of hydrochloric acid and thus all impurities were removed by washing with water. The composition was practically the same as previously found by Levene and Alsberg. The important point concerning the peptide part of the substance is its high nitrogen content, namely 17 per cent, which indicates a high proportion of the basic amino acids. Indeed, a rough estimation by the phosphotungstic acid method showed that 40 per cent of the total nitrogen belonged to the basic amino acids. On the basis of this consideration, it must be concluded that of the total nitrogen of the peptide, there was only 70 per cent in the form of amino groups. Hence, in the vitellinic acid there should be present one phosphoric acid residue to every two amino acids. Furthermore, if there is in the molecule only one definite amino acid to which the phosphoric acid is attached, then it should be possible to isolate it without much difficulty.

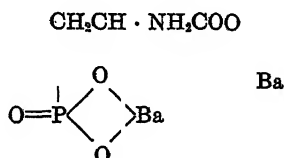
On dephosphorylation with  $0.25\text{ N}$  alkali the polypeptide is obtained in practically unchanged condition. It, similarly to the peptide part of vitellinic acid, contains 17 per cent nitrogen, and, similarly to the original vitellinic acid, yields on hydrolysis with

<sup>5</sup> Rimington, C., *Biochem. J.*, **21**, 272, 1179 (1927).

<sup>6</sup> Posternak, S., *Compt. rend. Acad.*, **186**, 1762 (1928).

mineral acid about 18 per cent ammonia. Of this not more than 25 per cent originates in amide nitrogen. This finding is in disagreement with the conclusion of Posternak and Posternak who thought the high ammonia formation is conditioned by the presence of the phosphoric acid residue. This high yield of ammonia may be regarded as an indication of the presence of a high proportion of serine in the molecule of vitellinic acid.

The most important fact to be reported in this communication is the isolation from the product of partial hydrolysis of vitellinic acid of a barium salt which had the composition of the salt of serinephosphoric acid.



The details of the preparation are given in the experimental part.

#### EXPERIMENTAL

*Preparation of Vitellinic Acid*—The procedure of Levene and Alsberg was followed in the main features. The ammoniacal solution was treated with picric acid and acetic acid was added to about pH 5 (calculated so that the proportion of ammonium acetate to acetic acid was 3:1 to 2:1). The crude vitellinic acid was precipitated with a minimum volume of alcohol. The crude substance was redissolved in water and reprecipitated with hydrochloric acid. The precipitate was washed free of picric acid and of ammonia and then dried by treatment with alcohol of progressively increasing concentration. The substance was practically insoluble in cold water, very slightly soluble in hot water, and the solution was acid to Congo red. On titration with thymolphthalein a solution of the substance consumes 2 equivalents of sodium hydroxide for each phosphorus atom, showing that in the region of pH 8, there are no titratable acidic groups in the substance and that only 1 equivalent of phosphoric acid is bound to the substance.



The proportions of nitrogen to phosphorus were as follows:

	N	P	N:P
First preparation.....	10.19	12.71	2.76
Second ".....	10.45	12.7	2.69
Third ".....	10.12	12.33	2.70

These values show a fairly constant composition and particularly a very constant relationship of nitrogen to phosphorus.

*Dephosphorylation of Vitellinic Acid and the Dephosphorylated Polypeptide*

It is known that dephosphorylation of all phosphoproteins proceeds very rapidly on the alkaline side and but very slowly in dilute acid. Thus, at pH 11.1 at 100° the substance loses 80 per cent of its phosphoric acid in 3½ hours, whereas in the presence of 2.0 N hydrochloric acid at the same temperature the loss in 10 hours is only 38 per cent. For the preparation of the polypeptide free from phosphoric acid, vitellinic acid was dissolved in a solution of 0.25 N sodium hydroxide and allowed to stand for 60 hours at 31°. To the solution ammonium acetate and magnesia mixture are added as long as a precipitate is formed. The filtrate from this precipitate is acidulated to pH 5, when the polypeptide settles out. To complete precipitation an equal volume of methyl alcohol is added. The precipitate is washed carefully with dilute methyl alcohol, faintly acidulated with acetic acid, and finally dried with methyl alcohol of progressively increasing concentration.

The nitrogen content of several samples of protein prepared in this manner is 17.48, 17.53, 17.56, and 17.28 per cent. The nitrogen content of the polypeptide of the vitellinic acid calculated from the nitrogen content of vitellinic acid likewise is 17 per cent, thus showing that on dephosphorylation the protein undergoes little change. The yield of the polypeptide was 70 per cent of the theory.

*Ammonia Formation on Acid Hydrolysis of Vitellinic Acid and of Its Protein Component*

For the purpose of comparing the ammonia formation of the two substances, each was hydrolyzed for 36 hours with 20 per cent

hydrochloric acid. The ammonia estimation was made by the method of Parnas.<sup>7</sup> The values for the vitellinic acid were found to be 17.3 per cent and for the dephosphorylated substance 17.9 per cent.

In order to determine the origin of the ammonia, the vitellinic acid was digested with 0.25 N sodium hydroxide solution for 60 hours at 31°. The yield of ammonia was 4.6 per cent of the total nitrogen. In order to test whether the treatment was sufficiently vigorous to hydrolyze all the amide nitrogen, asparagine was submitted to the same treatment, with a yield of 84 per cent nitrogen of the nitrogen present in the substance in the form of amide nitrogen. Thus, it is warranted to assume that not more than one-third of the ammonia developed on acid hydrolysis originated in the amide nitrogen of the polypeptide.

*Isolation of the Serinephosphoric Acid*—Vitellinic acid is hydrolyzed for 10 hours with 2.0 N hydrochloric acid. 38 per cent of the phosphoric acid is cleaved off. The solution is neutralized with barium carbonate and then made alkaline to phenolphthalein. About one-third of the bound phosphoric acid remains in solution and two-thirds, together with the inorganic phosphate, in the precipitate. This precipitate is dissolved in acetic acid and the solutions neutralized to litmus with ammonia water. A precipitate of inorganic phosphate with a small amount of organic phosphate is filtered off and the filtrate combined with the first filtrate is made alkaline to phenolphthalein by means of a solution of barium hydroxide. The substance is precipitated from the solution by means of 50 per cent alcohol. The precipitate is nearly completely soluble in a considerable volume of water. The aqueous solution is precipitated with 50 per cent alcohol. The ratio of N:P in this crude substance was 1.3:1; the ratio of NH<sub>2</sub> (Van Slyke):P is 1:1. The yield was 60 per cent of the phosphorus not cleaved off by hydrochloric acid.

For further purification the substance was redissolved in 2.0 N hydrochloric acid (an insoluble precipitate settled out which was removed) and the solution was allowed to digest an additional 3 hours. The solution was then neutralized to litmus, filtered from the greater part of the inorganic phosphates, and the filtrate was made alkaline with a solution of barium hydroxide. To the solu-

<sup>7</sup> Parnas, J. K., and Klisiecki, A., *Biochem. Z.*, **173**, 227 (1926).

tion alcohol is added to 50 per cent concentration. To remove all of the inorganic phosphate the precipitate is extracted with a large volume of water. To the aqueous solution containing nearly all the organic phosphate, alcohol is added to 25 per cent concentration. One part of the salt remains in solution, which, when precipitated with more alcohol has the composition of the dibasic salt of serinephosphoric acid.

5.400 mg. substance:	33.270 mg. $P_2O_5$
8.950 " "	: 0.353 cc. N at 29° and 753 mm.
0.0648 gm. "	: 0.0453 gm. $BaSO_4$
4.785 mg. "	: 1.915 mg. $CO_2$ and 0.930 mg. $H_2O$
$C_3H_5NO_5PBa$ . Calculated.	C 11.2, H 1.82, N 4.36, P 9.65, Ba 42.7
Found.	" 10.9, " 2.18, " 4.4, " 8.95, " 41.0

The material obtained by precipitation with 25 per cent alcohol was taken up in water. The greater part dissolved. Alcohol was then added to 50 per cent concentration and to the suspension a very dilute solution of barium hydroxide was added drop by drop to alkaline reaction to thymolphthalein. The precipitate was rapidly centrifuged and the centrifuge washed with 50 per cent alcohol, 90 per cent alcohol, and ether. Washing with alcohol of greater strength is not advisable. This substance had the composition of the tribasic salt of serinephosphoric acid. All of the nitrogen is present as  $NH_2$  nitrogen (Van Slyke).

$(C_3H_5NO_5PBa)_3$ . Ba. Calculated.	C 9.36, H 1.50, N 3.42, P 8.06, Ba 52.38
Found.	" 9.27, " 1.30, " 3.60, " 7.99, " 53.09

The optical rotation of the barium salt ( $c = 1.70$ ) dissolved in 10 per cent hydrochloric acid was  $[\alpha]_D^{25} = +0.14^\circ$ . The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{1 \times 1.70} = +8.2^\circ (\pm 0.02^\circ)$$

Calculated as the free acid, the rotation was

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{1 \times 0.81} = +17.3^\circ$$

# THE CONCENTRATIONS AND DISTRIBUTION OF POTASSIUM IN THE SERUM AND CELLS IN THE BLOOD OF NORMAL UNANESTHETIZED DOGS

By A. R. McINTYRE

(From the Laboratory of Pharmacology, Medical School, University of Michigan, Ann Arbor)

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Since Abderhalden (1), in 1898, published the results of analyses of the blood from various animals, there have been many determinations made upon potassium in dog serum. However, very few determinations have been made upon dog cells and serum simultaneously; and such determinations, including the work of Abderhalden, have been made apparently in the majority of cases upon blood collected in part or entirely under anesthesia either general or local. It became necessary in connection with another study to determine the distribution of potassium in the normal unanesthetized dog with considerable accuracy. This paper presents data from the examination of dog blood from normal males and normal (non-pregnant) females.

## Method

*Collection of Sample*—Six normal healthy<sup>1</sup> dogs and four non-pregnant bitches maintained on an adequate diet were used. Blood was drawn without anesthesia from an external jugular vein into Pyrex centrifuge tubes in quantities of approximately 50 cc. The blood was obtained at various periods in relation to the feeding time of the animals. If the animals struggled or became excited the blood was discarded. It has been shown by Doisy and Eaton (2) that variations in carbon dioxide tension have but an inconsiderable effect on potassium distribution in the

<sup>1</sup> Normal save for the presence of intestinal parasites in some of the animals.

blood; nevertheless, in some of the earlier determinations the precaution was taken of collecting the blood under oil. Experience showed, however, that loss of carbon dioxide was without effect upon the potassium concentrations. Moreover, the passage of oxygen through a sample for 10 minutes was likewise ineffectual in the modification of the ratio of cell to serum potassium although there was a manifest increase in oxyhemoglobin. Defibrination was accomplished by stirring with a glass rod. Greatest care was exercised to avoid hemolysis but many of the samples subsequent to centrifugalization showed slight hemolysis upon careful examination and were of no value. In the experiments upon the last four animals routine spectroscopic examination of the serum was made for hemoglobin. It was found that this method was little if any more critical than the simpler procedure of drawing a sample of serum into a glass tube about 15 cm. long and of 11 mm. bore and examining the serum longitudinally in the oblique light from a carbon arc. Samples of serum that appeared perfectly clear and free from hemolysis in the tonometer sometimes showed the familiar pink coloration due to hemoglobin when thus inspected.

*Preparation of Sample*—After centrifugalization for 1 hour the sera were separated from the cells and transferred to tonometers. The cells were rendered free from any remaining fibrin by the passage through coarse gauze while the transfer to the tonometers was performed. In the case of those samples collected under oil this was accomplished without loss of carbon dioxide.

*Sera*—1 cc. of serum was measured directly by means of a Van Slyke-Ostwald pipette into a platinum<sup>2</sup> crucible. A few drops of 4 N sulfuric acid were added and the crucible gently heated on a sand bath for from 3 to 4 hours until all fuming ceased. The residue was ashed directly by placing the crucible in an electric muffle for 12 hours or more at approximately 490°. Care was taken not to exceed this temperature by any considerable margin in order to avoid losses of potassium due to volatilization. The ash was always rendered perfectly white and free from carbon before proceeding. To the ash when cool were again added a few

<sup>2</sup> Quartz crucibles were found far from satisfactory because of the formation of "scale" and the subsequent impossibility of transferring quantitatively the precipitates to the filter.

drops of 4 N sulfuric acid and the crucible was again heated on the sand bath for from 2 to 3 hours. Ash so prepared was found to be free from ammoniacal compounds.<sup>3</sup>

*Cells*—A known weight of cells was hemolyzed by the addition of 15 parts of water. The proteins were then precipitated by means of the addition of 2.2 volumes of freshly prepared 20 per cent solution of trichloroacetic acid. Filtration was done through Whatman filter paper No. 50. The filtrates were clear and free

TABLE I  
*Concentrations of Potassium in Sera and Cells*

Dog No. and sex	Sera		Cells		$\frac{K_s \text{ (mm)}}{K_c \text{ (mm)}}$
	mg. per 100 cc.	$\frac{mM \text{ per kg.}}{H_2O^*}$	mg. per 100 gm.	$\frac{mM \text{ per kg.}}{H_2O^*}$	
1, ♂	25.2	6.98	23.3	9.10	0.77
2, ♂	19.7	5.42	24.5	9.59	0.56
3, ♀	22.6	6.15	30.1	12.10	0.51
4, ♂	20.0	5.52	28.7	11.24	0.49
5, ♀	21.3	5.82	27.4	10.70	0.55
6, ♂	21.6	5.95	31.6	12.31	0.48
7, ♂	21.0	5.78	26.8	10.43	0.55
8, ♀	20.5	5.63	25.1	9.79	0.57
9, ♀	20.2	5.52	25.4	9.92	0.55
10, ♂	23.3	6.42	32.5	12.80	0.50
Average...	21.54	5.92	27.04	10.80	0.55

\* Averages of four determinations.

from pigments. Aliquot portions of 5 cc. were placed in platinum crucibles, evaporated to a small bulk, and then ashed in a precisely similar manner to that for the sera.

*Method of Analysis*—The method employed was essentially that described by Shohl and Bennett (3). Only the minor modifications introduced will be described. They consisted of the evaporation *in vacuo* of the excess water subsequent to the addition of the chloroplatinic acid to the acidified ash but prior to the addition

<sup>3</sup> The author is indebted to Dr. Frank H. Wiley, National Research Fellow in Experimental Medicine, University Hospital, University of Michigan, for the suggestion of this method for the ashing of samples.

of the absolute alcohol (this was found to reduce a small loss occasioned by the slight solubility of potassium chloroplatinate in the aqueous solution of chloroplatinic acid) and the use of the modified Witt filtration apparatus for multiple determinations as described in another place (4).

Water determinations were made in duplicate on sera and cells by heating a known weight of cells or a known weight and volume of serum for 12 hours at  $110^{\circ}$  and reweighing.

*Accuracy of Method*—The method when tested upon aqueous solutions of known potassium content yielded results that agreed within less than  $\pm 1$  per cent of the theoretical value. When the method was applied to solutions prepared by the addition of known amounts of potassium chloride to previously analyzed serum, the error was greater and it is considered that the method has an accuracy of  $\pm 2$  per cent for biological fluids. (Determinations were made in quadruplicate. The average error for all determinations taken in sets of four was  $\pm 0.3$  per cent with a greatest error of  $+2$  per cent and least error of  $-0.1$  per cent.) Other methods of analysis were found, in our hands, not to exceed in accuracy the method employed and in general were more laborious.

*Results*—Table I contains the results from ten experiments. The abbreviations used are similar to those employed elsewhere (5, 6) and are self-explanatory. For ease of comparison the results are expressed both in mg. per 100 cc. of serum or 100 gm. of cells, and in mm per kilo of water. To arrive at the correct concentrations for 92 per cent of the animals, twice the standard deviation of the mean is employed, the standard deviation being 0.49, 1.26, and 0.08 for serum, cells, and ratios respectively.

#### DISCUSSION

It is obvious that the analysis of erythrocytes is subject to an error proportional to the amount of contaminating serum present. This has been pointed out by Kerr (7) who accordingly analyzed the serum and whole blood and by the aid of carefully performed hematocrit and specific gravity determinations attempted to calculate the potassium content of the cells. He rightly emphasized that in such a procedure the determinations of potassium in the whole blood and serum must be carried out by identical procedures; he thus precipitated both the serum proteins and whole

blood proteins and used the protein-free filtrates for examination. In many of our early experiments this technique was followed. It was found, however, that results with a much larger range of variation were obtained than by the more simple direct method. Furthermore, the unavoidable slight experimental errors involved in the obtaining of relative cell volume and the specific gravity of serum and cells had, in our hands, a cumulative error greater than that due to contamination of the cells by serum. The samples of serum when ashed directly gave higher values for their potassium concentration than when determinations were performed upon protein-free serum filtrates. This was not due to the presence of ammonia or its compounds. It would consequently appear that the precipitation of protein involved a loss of potassium; however, the loss was not constant in magnitude. The deviation from the mean value for potassium in the case of cells will be seen to be about twice that for the sera. Presumably this is partially accounted for by (1) the manipulative error involved in the precipitation of proteins from the cells, (2) variations in the amount of contaminating serum. Dill, Edwards, Florkin, and Campbell (8) in a recent paper find what appear to be somewhat lower concentrations of potassium in dog serum than are reported here. There is no ready explanation for this discrepancy. The values found in the present study are in general agreement with those of Abderhalden (1).

#### SUMMARY

1. In the normal unanesthetized dog the concentration of potassium in the serum is, for 92 per cent of the animals,  $5.92 \pm 0.98$  mm per kilo of  $H_2O$ .

2. In the cells for 92 per cent of such animals the concentration of potassium is  $10.79 \pm 2.5$  mm per kilo of  $H_2O$ .

3. The normal ratio  $\frac{K_s}{K_c}$  for 92 per cent of such animals is  $0.557 \pm 0.16$  subject to adjustments for the experimental error which is  $\pm 2$  per cent.

The author is indebted to Dr. R. G. Smith for many helpful criticisms and suggestions.



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## LACTOSE IN NUTRITION\*

By O. L. KLINE, J. A. KEENAN, C. A. ELVEHJEM, AND  
E. B. HART

*(From the Department of Agricultural Chemistry, University of Wisconsin,  
Madison)*

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In 1923, Dragstedt and Peacock (1) reported successful attempts to prevent the onset of tetany in thyroparathyroidectomized dogs by adding 50 to 100 gm. of lactose daily to the diet. This work followed reports of previous studies (2, 3) upon the influence of lactose on the bacterial flora and the reaction in the intestinal tract. To lactose has been ascribed the specific property of modifying the intestinal flora toward the aciduric rather than the proteolytic type. A somewhat similar, but less effective action has been attributed to dextrin (3); other carbohydrates seem to have no influence on the intestinal flora. Hudson and Parr (4), in their studies on the relation of the reaction of the intestinal contents to diet and flora, showed that lactose, in contrast to other carbohydrates, was responsible for the production of an aciduric flora and consequently an acid reaction in the intestine.

Inouye (5) extended the work of Dragstedt and Peacock and found that certain amounts of lactose and galactose in the diet served to prevent indefinitely the onset of tetany in dogs; when other forms of carbohydrate were used, the animals developed severe tetany within a few days. The fact that subcutaneous administration of lactose failed to prevent tetany suggested that the point of action was in the alimentary tract.

Bergeim (6), studying calcium and phosphorus utilization in rats, demonstrated that lactose, and to a lesser degree, dextrin, when present at levels of 25 to 50 per cent of the diet, produced a marked increase in calcium absorption, as contrasted with negative results

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obtained through the administration of glucose, sucrose, maltose, and starch. Bergeim used his iron index procedure in determining the amount of calcium absorbed. Robinson, Huffman, and Mason (7), working with male calves in balance experiments, showed that the ingestion of lactose favorably influenced the retention of calcium. From more recent work Robinson and Duncan (8) have concluded that increased calcium absorption is due to a specific property of lactose, rather than its influence in effecting an acid reaction in the tract. They based their conclusion upon pH measurements of the intestinal contents of rats, which showed that the change to an acid reaction due to lactose feeding occurs only in the lower part of the alimentary tract where very little or no absorption takes place.

That lactose feeding influences the intestinal flora in the chick is demonstrated by the favorable influence of lactose in preventing or curing coccidiosis. Beach (9) has shown that adding lactose to the chick ration leads to a pronounced acid reaction in the ceca.

A rather extraordinary influence of lactose has been recorded by Jarvis in infant nutrition (10). He made the following observation, "the tissues of these children (lactose) seemed more firm than those of the average infant receiving vegetable sugar, and resembled the condition of the breast-fed infant. Fat babies were rarely observed." In experiments with rats, Jarvis showed that the tissues from lactose-fed rats contained a greater amount of total solids than the tissues from rats receiving sucrose as their carbohydrate.

It is apparent that there is already in the literature evidence that lactose has certain nutritive properties not possessed by other sugars, and that it particularly influences both the reaction along the tract and the absorption of calcium. Pediatricians have made the notable observation that the breast-fed child rarely suffers from rickets. Hess (11) makes the following statement, "Breast feeding is a valuable measure for combating rickets, but should not be regarded as specific." In this connection it is significant to note that in human milk lactose constitutes from 50 to 55 per cent of the solids, while in cow's milk it constitutes generally about 40 per cent.

Although calcium absorption has been shown by several investigators to be influenced by lactose feeding, there has been no clear

cut demonstration of improved calcium utilization due to the action of this sugar. In this paper we wish to report results demonstrating the effect of lactose feeding upon calcium retention and utilization in rachitic chicks. The effect of lactose was determined by measuring the actual ash content of the skeleton, as well as the reaction of the intestinal tract. Chicks were chosen as the experimental animal because of their sensitivity to rickets and because the intestinal tract in this animal is large enough to furnish sufficient material for accurate pH measurements.

#### EXPERIMENTAL

Day old white Leghorn chicks were used in all of the work. They were divided into groups of ten each, and placed in pens equipped with wire screen bottoms and suitable warmers. The basal rachitogenic ration consisting of

Yellow corn.....	58
Standard wheat middlings.....	25
Crude casein.....	12
NaCl.....	1
Yeast (dry Northwestern).....	2
CaCO <sub>3</sub> .....	2

was fed *ad libitum* with distilled water. The chicks were weighed weekly. Previous work has shown that chicks receiving this ration alone develop definite symptoms of rickets in 5 to 6 weeks, and the ash content of the alcohol-extracted bones at this time is 26 to 28 per cent. When the ration is supplemented with vitamin D in the form of ultra-violet irradiation with a quartz mercury vapor lamp at a distance of 24 inches for 10 minutes daily, the chicks grow normally and the bone ash is about 40 per cent. One group receiving the basal ration alone, and one the basal ration plus irradiation were included in all of our experiments for comparison. In the first trial lactose, of U.S.P. quality, was added to the basal ration at 2, 5, 10, and 20 per cent levels, replacing in each case an equivalent amount of yellow corn. The chicks in all groups except the basal plus irradiation developed rickets, although the development was slightly delayed in the lactose groups, especially in those receiving 10 and 20 per cent. The average weight for each group at 6 weeks is given in Table I. It is readily seen that

the weight of chicks receiving lactose is somewhat greater than that of the chicks on the basal ration. Although growth on the 20 per cent lactose level was not as good as on the 5 and 10 per cent levels, it was considerably better than on the basal ration.

All the chicks were killed at 6 weeks of age and the tibia was removed from each individual for ash analysis. The bones were placed in 95 per cent ethyl alcohol until it was convenient to proceed with the analysis. They were then crushed, wrapped in filter paper, and extracted for 72 hours with hot 95 per cent alcohol. Next they were dried, ashed for 1 hour in an electric muffle furnace at a dull red heat, and the percentage of ash determined.

The results of the ash analyses are also included in Table I. The addition of lactose to the ration has a definite effect upon the

TABLE I  
*Record of Weight and Ash of Tibia in First Experiment*

	Basal	Basal + irradiation	Basal + 2 per cent lactose	Basal + 5 per cent lactose	Basal + 10 per cent lactose	Basal + 20 per cent lactose
Average weight in 6 wks., gm.....	135	340	150	205	190	177
Average ash content at 6 wks., per cent.....	27.5	40.7	28.9	29.7	31.5	32.7

ash content of the bones. The improvement over the basal group is very slight in the case of the 2 per cent addition. The degree of improvement increases as the level of lactose is increased. The usual spread in the bone ash figures between the basal group and the basal plus irradiation group is 12 to 15 per cent. The feeding of lactose at a 20 per cent level has caused an average increase in ash of about 5 per cent over the basal, which is one-third the total spread.

Noting the improvement due to the 20 per cent level of lactose we thought it important to feed a higher level of the sugar, and the results of eight complete experiments are found summarized in Table II. Since the chickens in some of the experiments were killed at 5 weeks and in other cases the experiments were not

terminated until the chickens were 6 weeks of age, the experiments have been separated into two groups dependent upon the length of time the animals were kept on the experiment. These experiments include beside the basal and basal plus irradiation groups, the basal plus 40 per cent lactose, the basal with 40 per cent lactose and vitamin D as ultra-violet irradiation, the basal plus 40 per cent maltose, and the basal with 5 per cent citric acid. Preliminary

TABLE II

*Records of Weight (Group Averages) and Ash of Tibia of Various Groups*

Age	Experiment No.	Basal group		Basal + irradiation		Basal + 20 per cent lactose		Basal + 40 per cent lactose		Basal + 40 per cent lactose + irradiation		Basal + 40 per cent maltose		Basal + 5 per cent citric acid	
		Weight		Weight		Weight		Weight		Weight		Weight		Weight	
		gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent
6	3	110	26.3	305	40.4	195	31.2	160	39.0						
6	5	142	28.5	350	39.6			170	35.2						
6	7*	150	27.8	375	40.9			210	39.6	310	47.4	140	28.8	139	28.6
6	10	140		360				210							
Average		135	27.5	338	40.2			188	37.9						
5	2	105	26.5	200	39.5	111	32.1								
5	6	110	25.4	200	41.8							125	25.6		
5	8	110	25.5	330	41.4			195	35.9	200	44.4				
5	9	120	28.2	270	41.3			150	33.9	220	42.1				
Average		111	26.4	245	41.0			172	34.9	210	43.3				

\* In Experiment 7 the lactose used was ether-extracted.

work showed that it was not advisable to add 40 per cent of the sugar to the ration for day old chicks. Therefore, the introduction of lactose was a graduated one; 10 per cent was introduced in the ration the 1st week, 20 per cent in the 2nd week, 30 per cent in the 3rd week, and 40 per cent thereafter. Further, the displacement of the large amount of yellow corn by the 40 per cent level of sugar necessarily reduced the protein content of the ration, as well as the amount of vitamin A present. Consequently, the protein level was kept equivalent to that of the basal ration, about 19 per

cent, by the addition of the proper amount of casein. The lowered supply of vitamin A was similarly equalized by the addition of 1 per cent of dried yellow carrots.

In Experiment 3 where the 40 per cent of lactose was fed, an almost normal bone ash was obtained, 39.0 per cent as compared to 40.4 per cent in the group receiving sufficient vitamin D. Here again, *though the growth was not normal* in the lactose group, the average weight for the birds in this group was 50 gm. higher than that for the basal group at 6 weeks of age. All further data obtained by feeding the 40 per cent lactose substantiated the results obtained in Experiment 3.

In order to determine whether the absence of vitamin D was the factor responsible for retarded growth on the 40 per cent lactose ration, groups of chicks receiving both 40 per cent of lactose and irradiation were included in some of the experiments. In these groups (Experiments 7 to 10) the growth was slightly better than was obtained when lactose was the only addition, but even with both additions the animals did not grow as well as those receiving irradiation alone. The increased water consumption and the catharsis caused by the high level of lactose in the ration are probably the factors responsible for the retarded growth.

The ash content of the bones from the irradiated chicks receiving the lactose ration was distinctly higher than that from the irradiated chicks receiving the basal ration alone. This indicates that even in the presence of sufficient vitamin D, the addition of lactose improves the absorption of calcium. In other words, lactose acts as a supplement to vitamin D in calcium assimilation.

In order to compare the activity of lactose with the action of another disaccharide, a Pfanstiehl preparation of maltose was added to the basal ration at the 40 per cent level. The figures obtained for growth and bone ash in Experiments 6 and 7, where maltose was fed, show that this sugar was without effect.

To find whether or not a relatively insoluble organic acid would help to increase calcification on the rachitogenic ration used, in a manner similar to lactose, citric acid was added to the basal ration at a level of 5 per cent. Like maltose, this acid had no influence on growth or per cent ash of the tibia. This is concluded from the figures obtained in Experiment 7 (Table II).

To dispel any question of the possibility of lactose furnishing

vitamin D, which might be responsible for the improved bone formation, an ether extraction of the lactose was made. The sugar was extracted for 3 days with ethyl ether in a large Soxhlet extractor, the ether carefully removed, and the sugar fed at the 40 per cent level. The results secured with the group of chicks in Experiment 7 show that the extraction did not reduce the effect lactose has upon calcium utilization. Data for Experiments 8, 9, and 10 give further evidence of an improved calcium absorption and bone formation due to the presence of lactose. This is conclusive evidence that lactose has a decided effect upon calcification in rickets. An attempt has been made to explain this action by studying the pH of the intestinal tract from the chicks used in these experiments.

### *Intestinal Reaction*

For this work the quinhydrone electrode was used. Robinson and Duncan (8) have compared the quinhydrone with the hydrogen electrode in determinations of this type, and found the results in close agreement.

Immediately after killing the chicks, by separating the cervical vertebra with sudden tension, the intestine was removed and divided into four equal segments. The large intestine, which is a very small part of the intestinal tract, was disregarded, and the division into segments was an arbitrary one. The first segment taken was the loop adjacent to the pancreas. The fourth segment was adjacent to the cecum, and ended just above the cloaca. The remaining intermediate portion of the intestine was divided into two equal segments. The contents of each segment were stripped into separate beakers and diluted to 20 cc. with neutral water prepared by distillation in a closed glass system. The readings were made immediately at room temperature with a Leeds and Northrup potentiometer against a saturated calomel half-cell, a saturated KCl-agar bridge being used. Grayzel and Miller (12), when determining the pH of the intestinal contents of dogs, made a series of determinations on diluted and undiluted samples and found that dilution did not materially alter the pH. This confirmed the observation of Davidsohn (13) and others that in the case of heavily buffered solutions of low acidity, dilution has no appreciable effect on the pH. In practically all cases the method



used for obtaining the sample was found to be satisfactory. When the chicks were allowed to reach the stage of severe rickets, and were too weak to consume much of the ration, there was some difficulty in obtaining sufficient material to work with. Also in a few cases even in normal chicks the first segment of the intestine contained too small an amount of material for a determination. One outstanding effect of high lactose feeding was the large amount of fluid material in the intestine and ceca, due undoubtedly to the greater water consumption.

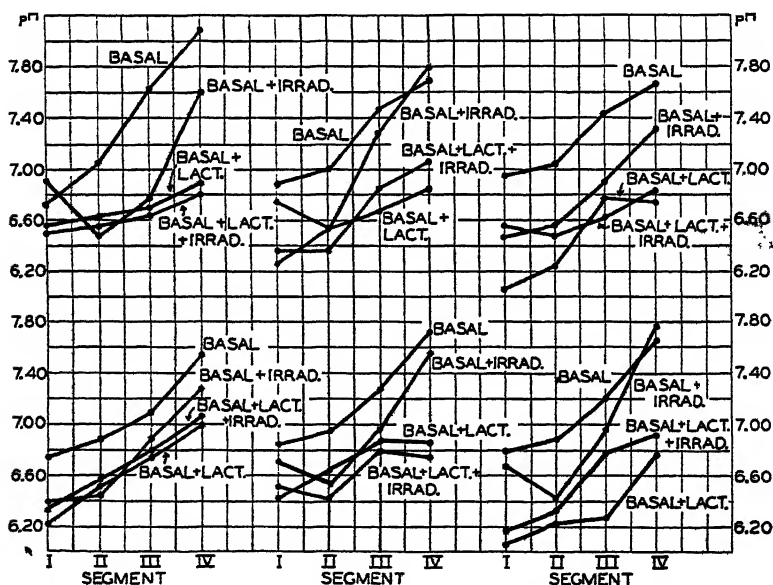


CHART I. Effect of diet on intestinal reaction. Segments I, II, III, and IV represent the four levels of the intestine used.

Typical pH data have been plotted in Chart I. To avoid confusion, data from only four of the rations used are plotted together. These, however, are sufficient to bring out the effect of lactose feeding on the reaction in the tract. Of the lactose-fed chicks, only those receiving the 40 per cent level of sugar were used for the data presented in this paper. The curves in Chart I are those for typical individual cases in their respective groups, and do not represent averages. Attention should first be called to the differ-

ences between individuals fed the basal ration, without vitamin D, and those receiving lactose. In the first segment, the duodenal loop about the pancreas, the reaction in the lactose-fed chicks is generally more acid than that from the chicks fed the basal ration, though the degree is variable. Other factors, such as the acid from the stomach, and the bile, are probably affecting the reaction in that segment. In the birds receiving the basal ration the reaction steadily increases from a point near 6.80, becoming more alkaline as the material passes through the tract. In contrast to this curve is that for the lactose-fed chick, starting lower, at a pH varying from 6.10 to 6.60, and rising very much more slowly. In only a few cases does the reaction in any part of the tract reach a pH above 7.10. Addition of vitamin D does not influence the reaction markedly in the presence of a high level of lactose. The reaction curve for chicks receiving vitamin D (ultra-violet irradiation) is interesting in that it is in the acid range where most active calcium absorption takes place, and decidedly on the alkaline side in the lower part of the tract. The curves clearly demonstrate that lactose is effective in maintaining an acid reaction throughout the length of the tract, and particularly in the second and third segments where active absorption undoubtedly takes place.

#### DISCUSSION

It is well known since the work of Zucker and Matzner (14) that the ingestion of vitamin D leads to a more acid reaction along the intestine. Grayzel and Miller (12) succeeded in lowering the reaction in the tract of dogs from pH 6.59 to 7.44, on a rachitogenic diet, to 5.91 to 6.84 by supplying either ultra-violet irradiation or cod liver oil as a source of vitamin D. Whether this change in reaction is the cause of increased calcium absorption or the result of removal of calcium from the tract, is questionable. That lactose is responsible for some change in intestinal absorption has been demonstrated. The increased calcification obtained with lactose feeding in the presence of sufficient vitamin D may indicate that two factors are affecting calcium absorption, the action of vitamin D and the solubility due to higher acidity. This question can only be answered by further investigation. Chicks fed a rachitogenic ration supplemented with 40 per cent lactose exhibited no gross symptoms of rickets at the age of 6 weeks, at which time

most of the birds on the basal ration alone had succumbed. The lactose-fed birds were as active as those receiving ultra-violet irradiation, although they had not grown so well.

While it is well known that chicks become decidedly rachitic upon a liquid milk-grain ration, or a grain-skim milk powder ration, yet in such cases the lactose is very much less than 40 per cent of the ration. It is probably lower than 20 per cent of the solids. It has been shown that not until high lactose feeding was practiced was there a decided influence upon calcium absorption and skeletal development. It is for this reason, probably, that human milk with 55 per cent of the solids as lactose acts favorably in lowering the incidence of rickets in breast-fed children.

Evidence has been presented to show that lactose causes a marked lowering of the reaction throughout the length of the tract, when used to supplement a rickets-producing ration. Whether the resulting acidity is responsible for increased calcium absorption has not been definitely shown. We reserve for future work a more complete interpretation as to how lactose functions.

#### SUMMARY

1. Lactose fed to young chicks at a level of 40 per cent of a rachitic ration (air-dried weight) had a very favorable effect upon calcium absorption and skeleton building, as well as a positive influence in maintaining the reaction along the entire length of the intestinal tract more acid than on the basal ration. However, with lactose feeding, in the absence of vitamin D, growth was subnormal.

2. Lactose fed at a 20 per cent level improved calcification to a smaller extent. At still lower levels, 2 to 10 per cent, the effect was very slight, but proportional to the amount of sugar fed.

3. Maltose fed at a 40 per cent level, or citric acid at a 5 per cent level, had no effect whatever on calcium absorption or intestinal reaction.

4. Ultra-violet irradiation of the birds fed the rachitic ration caused a distinct increase in acidity in the upper part of the intestine; but the lower part was affected to only a slight extent. Addition of lactose either alone, or with vitamin D, increased the acidity throughout the entire tract.

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## PENTOSE METABOLISM

### I. THE RATE OF ABSORPTION OF *d*-XYLOSE AND THE FORMATION OF GLYCOGEN IN THE ORGANISM OF THE WHITE RAT AFTER ORAL ADMINISTRATION OF *d*-XYLOSE\*

BY MABEL M. MILLER AND HOWARD B. LEWIS

(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)

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Xylose, first discovered and isolated from wood by Koch in 1881 (1), has been one of the rarer sugars, since it could be prepared only with difficulty and at considerable cost. In 1930, however, the United States Bureau of Standards in a cooperative investigation announced a simple method of preparation of xylose (2) from cottonseed hull bran, a very cheap raw material. Xylose thus becomes a sugar obtainable at a price comparable to that of sucrose. The wide publicity given to this announcement of the cheap production of xylose on a semicommercial scale has led to statements concerning xylose in the current popular literature which may be misleading. Thus xylose has been characterized (3) as a "slenderizing sugar from cottonseed" and, in the same article, as a "non-fattening sugar." Since in popular usage the term sugar is usually considered to imply a substance utilizable as a food by the higher forms of animal life, it becomes of importance to raise again the question of the rôle of xylose (and the pentoses) in nutrition. Early in the progress of the work, through the courtesy of Professor James L. Kassner of the University of Alabama and of the Bureau of Standards, a supply of this new product was placed at our disposal for a study of this problem.

Many conflicting conclusions have been drawn by various workers concerning the extent and mechanism of the utilization of the pentoses. The work has been limited hitherto by a lack of uni-

\* A preliminary report of a part of this investigation appears in *Proc. Soc. Exp. Biol. and Med.*, **28**, 448 (1930-31).

formity in the experimental procedure, by inadequate analytical methods, and by the cost of the pentoses to be studied. Pfüger (4) in 1905 reviewed and severely criticized the older work which indicated that pentoses could give rise to glycogen in the animal body and concluded that no adequate experimental evidence was available in support of this belief. Of later workers in this field, Brasch (5) observed no glycogen formation in dogs or rabbits after oral administration of rhamnose, arabinose, or xylose. On the other hand, Thomas, Gradinescu, and Imas (6) in experiments with frogs and Nitzescu and Benetato (7) in experiments with rabbits observed an increased glycogen storage after the administration of the five carbon sugars. In agreement with older work, the most effective source of glycogen derived from pentoses was rhamnose.

This brief review serves to illustrate the contradictory nature of the evidence concerning the transformation of the pentoses into glycogen. In these experiments, the species of experimental animals were frequently different, the amounts of pentose fed varied, the preliminary treatment of the animals was not uniform, and no knowledge as to whether the pentose fed was actually absorbed from the intestine was available. It was impossible to compare absorption of and glycogen formation from the pentoses with those of a readily utilizable sugar such as glucose.

By the new method of study introduced by Cori (8), it has become possible to determine with considerable accuracy not only the extent of absorption but also the rate at which absorption has occurred. With such additional data available, studies of glycogen formation assume a greater significance. In the present paper the rate of absorption of xylose from the intestinal tract of the white rat has been studied by the procedure of Cori (8) and in the same animals, the extent of glycogen deposition has been determined. Further studies of the fate of the xylose absorbed will be presented in a subsequent paper.

#### EXPERIMENTAL

Young white rats weighing from 100 to 200 gm. were fasted for 24 hours. A known amount of xylose (determined by the technique employed by Cori) was introduced by stomach tube and after the desired interval of time, the animals were killed with chloroform. The entire gastrointestinal tract was exposed, ligated

at each end, and removed. The tract was split open and the contents were washed out with several portions of hot water into a volumetric flask of 100 cc. capacity. After cooling, 5 cc. of 10 per cent sodium tungstate and a like amount of 0.66 N sulfuric acid were added, the contents of the flask were diluted to the mark with water, and the precipitated proteins were filtered off. The filtrates were used for the determination of the amount of unabsorbed pentose.

Xylose was determined by the method of Hagedorn and Jensen. Since the values obtained in this method are given in terms of glucose, it was necessary to determine the reduction effected by known amounts of xylose. After these values had been obtained and plotted, a table of reduction values in terms of xylose was constructed from the graph and these figures were used in subsequent determinations.

One group of seventeen rats served as controls for determining the normal reduction values of the contents of the gastrointestinal tract after a 24 hour fast. Values ranging from 8.3 to 16.3 mg. with an average value of 11.2 mg. (calculated as xylose) were obtained. In the xylose feeding experiments, all values for residual sugar in the gastrointestinal tract were accordingly corrected for the normal fasting reduction value equivalent to 11 mg. of xylose.

In a series of check experiments, the accuracy of the experimental procedure was tested as follows: Rats were fed xylose in the usual manner (approximately 2 cc. of a 50 per cent solution of xylose), killed immediately without allowing any time interval for the absorption of the carbohydrate, and the contents of the tract were analyzed. In ten experiments in which amounts of xylose varying from 700 to 1030 mg. were fed, the absolute error in the amount of xylose recovered varied from +19 to -21 mg., with an average percentage recovery of 99.5 per cent of the xylose fed.

Most of the rats used for the determination of the absorption coefficient were used also for glycogen estimations. Therefore, after the removal of the gastrointestinal tract from 5 to 10 minutes were needed for the preparation of the tissues for glycogen analysis. In some cases the analysis of the gastrointestinal contents was begun within 10 to 15 minutes after the death of the rat, but in most instances, the gastrointestinal tracts as removed from the animals were placed in covered beakers and kept in the ice box



TABLE I

*Rate of Absorption of Xylose and Glucose and Formation of Glycogen after Oral Administration of Xylose and Glucose*

Sugar administered	Rat No.	Weight after 24 hr. fast	Absorption period	Sugar fed	Sugar absorbed per 100 gm. rat per hr.	Glycogen	
						Liver	Body
	Average of 11 rats (controls)	gm.	hrs.	mg.	mg.	per cent	per cent
		103		0	0	0.11 (0.07-0.17)*	0.05 (0.04-0.08)
Xylose	Average of 6 rats	169	1	520-930	29 (25-34)	0.16 (0.09-0.27)	0.05 (0.05-0.06)
"	" "	149	2	530-1010	39 (29-54)	0.13 (0.09-0.19)	0.06 (0.04-0.08)
Xylose	19	94	3	570	74	0.13	0.05
	20	107	3	570	50	0.15	0.07
	21	106	3	570	56	0.16	0.08
	23	108	3	390	34	0.19	0.07
	25	111	3	390	33	0.16	0.06
	26	123	3	390	33	0.14	0.07
	27	129	3	560	49	0.11	0.04
	28	116	3	560	43	0.15	0.06
	29	137	3	560	41	0.17	0.05
	30	122	3	560	38	0.15	0.05
	31	103	3	560	52	0.08	0.07
	32	118	3	560	54	0.06	0.04
Average.....		114			46	0.14	0.06
Glucose	47	118	3	725	200	2.00	0.15
	50	96	3	725	231	2.00	0.19
	95	154	3	800	144	1.70	0.10
	96	151	3	800	147	1.54	0.10
	97	147	3	800	151	1.37	0.11
	98	159	3	800	149	1.18	0.10
	103	150	3	890	165	3.08	0.26
	104	158	3	890	145	2.91	0.24
Average.....		141			166	1.97	0.16

\* The figures in parentheses indicate the maximal and minimal values observed in each series.

TABLE I—*Concluded*

Sugar administered	Rat No.	Weight after 24 hr. fast	Absorption period	Sugar fed	Sugar absorbed per 100 gm. rat per hr.	Glycogen	
		gm.	hrs.			Liver	Body
Glucose†	145	130	3	177	44	0.54	0.09
	146	139	3	177	42	0.56	0.08
	147	131	3	177	44	0.63	0.09
	149	168	3	226	44	0.57	0.08
	150	174	3	226	42	0.57	0.08
	151	175	3	226	42	0.59	0.08
	154	169	3	266	52	0.69	0.09
	155	163	3	266	54	0.64	0.10
	156	166	3	266	52	0.75	0.10
	157	179	3	266	49	0.72	0.10
	158	174	3	266	50	0.66	0.10
Average.....		160			47	0.63	0.09

† The animals in this series received glucose in such an amount that the absorption in 3 hours is comparable to the absorption of xylose during the same time interval.

until all the rats for that day had been killed. When this procedure was followed, analysis of the gastrointestinal contents was begun within 1.5 to 2 hours after the death of the rat. No noticeable differences in the xylose content within the same groups were found due to the longer time interval before analysis was begun. Therefore, it can be assumed that bacterial action, if any took place, was too small to be significant.

As in previous experiments from this laboratory (9), in the glycogen determinations (Pfüger), the glucose formed by the hydrolysis of the glycogen was estimated by the method of Hagedorn and Jensen and the factor of Pfüger (0.927) was used for the conversion of glucose into glycogen. In Table I, under the heading "body glycogen" is given the glycogen content of the entire organism minus the liver and gastrointestinal canal.

The xylose as received from the laboratories of the University of Alabama and the Bureau of Standards was slightly yellow in color. This product was recrystallized twice from hot ethyl alcohol to which a small amount of norit was added. The final white product

was dried in a vacuum oven at 40° and then in a desiccator over calcium chloride.

#### DISCUSSION

The experimental groups consisted of six series of animals, a fasting control group of eleven rats, three groups which were killed 1, 2, and 3 hours respectively after the administration of the xylose, and two control groups which received glucose and were killed after 3 hours. Since the results of the 3 hour absorption periods were considered most significant, the figures for these groups alone are presented in detail in Table I, the results with the other groups being summarized.

The absorption coefficients of xylose showed a tendency to increase as the absorption period was prolonged. Average values of 29, 39, and 46 mg. of xylose absorbed per hour per 100 gm. of rat were observed during absorption periods of 1, 2, and 3 hours respectively. We found it impractical to continue absorption studies beyond 3 hours because of the frequent development of diarrhea. Cori (8) obtained an absorption coefficient of 62 mg. in the 1st hour and a lower but nearly uniform rate of absorption subsequently, 24, 28, 32, and 28 mg. at the end of absorption periods of 2, 3, 4, and 5 hours duration respectively. In none of our group of six animals did the rate of absorption in the 1st hour approach that found for the same period by Cori. Macleod, Magee, and Purves (10) were able to show that when concentrated solutions of glucose were fed, they were diluted in the stomach by the gastric secretion to a concentration of approximately 0.75 M before being discharged into the intestine and that the rate of discharge of such sugar solutions through the pylorus was inversely proportional to their concentration. If these findings are applicable also to xylose, a slower absorption of xylose during the 1st hour after feeding might have been expected in both Cori's and our own experiments, since the solutions of xylose fed were much more concentrated than 0.75 M (approximately 1.5 to 3.0 M in our series) and some delay in discharge through the pylorus would presumably result because of the period required for dilution. Our results, in general, lend support to the findings of Macleod and his coworkers. We have also observed that our

animals, which were allowed free access to water at all times, drank frequently after the feeding of the concentrated solutions of the sugars.

Inspection of the data concerning the glycogen content of the liver and the rest of the body, fails to reveal any appreciable increase in glycogen content after xylose feeding as compared with the fasting controls. On the other hand, when glucose was fed in amounts similar to xylose, the deposition of glycogen was marked both in the liver and in the rest of the body. However, since the rate of absorption of glucose was so much higher than that of xylose, it was considered necessary as a final control to investigate the glycogenetic value of glucose, when this sugar was fed at such a level that the amount absorbed in 3 hours would approximate the amount of xylose absorbed in the same period. In such experiments the amounts of carbohydrate available for glycogenesis would be approximately the same for both glucose and xylose. In this series (Table I) the average rate of absorption per 100 gm. of rat per hour was 47 mg. of glucose, a figure comparable to the average value of 46 mg. in the 3 hour absorption experiments with xylose. From a comparison of the glycogen values with those of the control and of the 3 hour xylose absorption periods, it is evident that a very considerable formation of glycogen has occurred even with smaller amounts of glucose.

When such smaller amounts of glucose were fed, a large part of the glucose was undoubtedly absorbed very promptly. It was felt that the greater glycogen formation from glucose fed at the level of xylose absorption, as compared with the xylose experiments, might have been due to this more rapid absorption of glucose with the resulting longer period for glycogen formation. To rule out this possibility, Rats 145, 146, and 147 received the glucose in three doses at hourly intervals, thus affording glucose for absorption over the entire 3 hour period. There were no appreciable differences in either liver or body glycogen between these animals which received glucose in fractional doses and those which received the entire amount of glucose in a single feeding. The lack of significant deposition of glycogen after xylose feeding can hardly be due to the slow absorption of this sugar but must in all probability be related to the difficulty of utilization of the xylose.

## SUMMARY

1. The absorption of xylose from the gastrointestinal tract has been determined by the method of Cori (8) in the white rat. In confirmation of the work of earlier investigators, xylose was absorbed more slowly than glucose. The rate of absorption under the experimental conditions used was lower during the 1st hour than in subsequent hours, absorption coefficients of 29, 39, and 46 mg. per 100 gm. of rat per hour being obtained for absorption periods of 1, 2, and 3 hours duration.

2. No evidence was obtained that xylose fed under our experimental conditions was a significant source of glycogen in the white rat. A marked glycogenesis was observed in experiments in which glucose was fed in comparable amounts and under similar conditions. These studies afford no evidence of utilization of xylose in the rat.

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## PENTOSE METABOLISM

### II. THE PENTOSE CONTENT OF THE TISSUES OF THE WHITE RAT AFTER THE ORAL ADMINISTRATION OF *d*-XYLOSE

BY MABEL M. MILLER AND HOWARD B. LEWIS

(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)

(Received for publication, July 8, 1932)

In the preceding paper (1), a study has been made of the rate of absorption of xylose from the gastrointestinal tract of the young white rat by the method of Cori and of the formation of glycogen after xylose feeding. The xylose was shown to be absorbed readily, but at a rate much less rapid than was glucose. Under the experimental conditions, no formation of glycogen could be demonstrated after the oral administration of xylose. In an attempt to gain further information as to the fate of the xylose absorbed, we have been concerned with the changes in the pentose content of the tissues (liver, kidney, muscle, blood) after the feeding of xylose.

#### EXPERIMENTAL

The experimental animals, young white rats, were fasted for 24 hours and were then fed xylose (2 cc. of a 50 per cent solution) as already described (1) in the preceding paper. After an absorption period of 3 hours, the animals were killed by chloroform. The liver, kidneys, and muscles of the hind legs were removed with as little injury to the tissues as possible, rinsed with a very small amount of water to remove any surface blood, wiped dry with filter paper, and weighed as rapidly as possible.

Protein-free extracts for the determination of pentoses were prepared as follows: Liver or kidney tissue was minced with scissors and ground in a mortar with clean quartz sand and 8 cc. of 10 per cent trichloroacetic acid. The extract obtained was decanted through a small filter paper into a 50 cc. volumetric flask and the grinding and extraction were repeated twice with fresh trichloro-

acetic acid, a total of 25 cc. of the acid being used. The mortar was then rinsed twice with 8 cc. of water and the rinsings were transferred to the filter. The filter paper was washed with water to give a final volume of 50 cc. in the volumetric flask. The procedure with muscle tissue was similar except that the minced tissue was ground with sand and 10 cc. of water before the addition of the first portion of trichloroacetic acid. The entire liver, both kidneys, and samples of approximately 2 gm. of muscle were used for the analyses.

The blood (removed by puncture from the heart) was transferred to a 50 cc. volumetric flask containing approximately 15 cc. of water. To the hemolyzed blood, 25 cc. of the trichloroacetic acid were added slowly, the flask was shaken vigorously, the contents were diluted to the mark with water, shaken, and the precipitated proteins were removed by filtration.

Pentoses were determined in the protein-free extracts by the colorimetric method of McCance (2), modified slightly because of the presence of trichloroacetic acid in our filtrates. In routine determinations, 4 cc. of protein-free filtrate from liver were measured into a Pyrex test-tube and 2 cc. of water and 3 cc. of concentrated hydrochloric acid were added. In the standard tube were placed 2 cc. of a xylose solution containing 0.1 mg. of xylose per cc., 2 cc. of 10 per cent trichloroacetic acid (since each 4 cc. of the tissue extract contained this amount of trichloroacetic acid), 2 cc. of water, and 3 cc. of concentrated hydrochloric acid. In the case of filtrates from kidney, muscle, and blood, the pentose content was usually too low to give sufficient color in the reaction to read satisfactorily. To 4 cc. of these filtrates 2 cc. of the standard xylose solution (0.2 mg. of xylose) were added in place of the water.

The Pyrex test-tubes containing the unknowns and the standards were fitted with reflux condensers consisting of 24 inch glass tubes inserted through tin-foil covered corks. The tubes containing the condensers were placed in a boiling water bath for 2 hours. After removal from the water bath, the tubes were cooled and 4 cc. of benzene were added to each. The tubes were then corked and shaken vigorously for 3 minutes in order to extract the furfural into the benzene layer. The tubes were allowed to stand for 30 minutes to get a complete separation of the benzene layer from the water layer, 2 cc. of the benzene layer were carefully removed, and 3 cc.

of the benzidine solution were added to develop the color. If any water became mixed with the benzene, the color would not develop completely. To further protect against the presence of moisture, all test-tubes and corks used for aliquots of the benzene layer were dried in the oven and kept in a desiccator until used. In spite of these precautions, great difficulty with the fading of the colors or with the lack of complete color development was experienced. The cause was found to lie in impurities present in some of the reagents used. Therefore, all reagents were purified. The trichloroacetic acid was distilled twice, the benzidine was recrystallized twice from water-free benzene, dried at 40°, and kept in a desiccator until needed. Thiophene-free benzene was used and a satisfactory lot of glacial acetic acid was found by testing the various samples available in the laboratory. The absolute alcohol and glacial acetic acid were tested with anhydrous copper sulfate for the presence of water and were not used if any blue color developed. After all of these precautions had been taken, the color developed fully within 15 minutes and showed no evidence of fading throughout a 2 hour period.

The accuracy of the method was tested with xylose solutions of known concentration and with mixtures containing xylose and glucose. The best proportionality was obtained with solutions of such a strength that the colorimetric reading was slightly lower than that of the standard, but good proportionality was secured with solutions of widely different strength. No interference in the development of the color resulted from the presence of glucose. It was also necessary to determine whether xylose added to tissue extracts could be recovered satisfactorily since xylose was added to certain of the tissue extracts in order to obtain a color of sufficient intensity to permit accurate comparison in the colorimeter. A typical series of control experiments of this kind may be cited. The pentose content of 2 cc. of a trichloroacetic acid filtrate of liver was found to be 0.157 mg. When 0.05, 0.10, and 0.15 mg. of xylose were added to 2 cc. portions of this filtrate, the pentose determinations showed 0.204, 0.259, and 0.308 mg. respectively or a pentose content of the original filtrate of 0.154, 0.159, and 0.158 mg. respectively as compared with the value of 0.157 mg. for the filtrate to which no xylose was added. That is, the pentose content of the liver extract was essentially the same regardless of



whether xylose was added to the extract. While we realize that the addition of known amounts of xylose to the unknown and the calculation of the xylose content of the tissue extracts by difference are not desirable procedures, we believe that the results of a long series of similar check experiments justify this, particularly since in small animals such as those used here, the amount of material available for analysis is small. Moreover, the difference in the analytical results between our control and our experimental animals is clearly beyond the possible error of the method.

It is recognized that the values obtained by this procedure cannot be interpreted strictly as pentoses. Glycuronic acid gives rise to furfural on treatment with acid. However, in a trichloroacetic acid filtrate from tissues, glucoproteins should not be present and glycuronic acid should exist only in the form of free chondroitic acid or similar substances or as conjugated glycuronates. Furfural may also arise from combined pentoses of tissues. Nucleoproteins should not occur in our protein-free filtrates, but the presence of nucleotides and nucleosides appears probable. The presence of nucleotides (*e.g.* adenylic, guanylic, and inosinic acids) in blood and tissues has frequently been demonstrated. Nucleotides of different origin vary greatly in their stability. Thus muscle adenylic acid (3) has been shown to yield furfural in traces only by the pentose method of Hoffman (4), while yeast adenylic acid yields furfural abundantly (3, 4). The pyrimidine nucleotides are relatively stable and yield little furfural, while the purine nucleotides and nucleosides (from yeast) yield furfural almost quantitatively (4). We have observed a similar ease of formation of furfural from the pentose present in yeast nucleic acid and adenine mononucleotide from yeast<sup>1</sup> in the McCance pentose method as used by us. It is probable that some of the pentose measured in our determination may have originated from non-pentose precursors and that some may have been combined as nucleoside or nucleotide. We have felt it permissible to speak of our results in terms of "pentose" or of "xylose" since our chief interest lies in the comparison of the results obtained in control experiments and in experiments in which xylose was fed.

<sup>1</sup> We are indebted to Dr. H. O. Calvery for supplying us with these substances for a study of their behavior in the method for pentose determination

## DISCUSSION

The experimental results obtained with rats fasted 24 hours and with rats fed xylose 3 hours prior to death are presented in Table I. In the fasting control animals, the pentose content of the tissue extracts was higher in muscle tissue than in liver or kidney. We have been unable to find data on the pentose content of tissues which are comparable to our own. Direct comparison with the few values in the literature cannot be made since in our experiments, the analyses were made on protein-free extracts of the tissues, whereas the older determinations were made on the hydrolysis products of the entire tissue. Moreover, Bendix and Ebstein (5) dried the tissues prior to analysis by shaking with alcohol, ether, and acetic acid, a procedure which might have been expected to remove soluble pentoses either free or in combination. In general, our values for liver and kidney, as might be expected, are lower than those previously recorded for the total pentose content of tissues (5-8). In the case of muscle tissue, which contains such soluble substances as inosinic and adenylic acids, our values were higher than those obtained on washed tissue.

In the xylose feeding experiments, after an absorption period of 3 hours, the pentose contents of the liver and kidney were greatly increased (Table I). The experimental values indicate definite increases since in every case the minimal values for these tissues of the xylose-fed rats were greater than the maximal values of the control series. The range was greater in the values obtained for the kidney than for the liver. This is probably to be explained by the accumulation of the pentose in the kidney prior to excretion in the urine, since it has long been recognized that after ingestion of pentoses, an excretion of these carbohydrates occurs. In contrast to those of the liver and kidney, the pentose contents of the muscles were practically identical in the control animals and in the animals receiving xylose. Cori and Goltz (9) have shown that arabinose penetrates muscle tissue more slowly than liver, a finding in harmony with the results of these experiments.

In order to demonstrate that these changes in the pentose content of the tissues were due to the pentose fed, a control series was studied in which 2 cc. of a 50 per cent solution of glucose were fed in place of xylose. In contrast to the experiments just discussed,

the results (Table II) showed clearly that the administration of glucose did not alter the pentose content of the tissues.

TABLE I

*"Pentose" Content of Liver, Muscle, and Kidney of White Rats after a 24 Hour Fast and after Oral Administration of Xylose*

All results are calculated as mg. of xylose per 100 gm. of tissue.

	Rat No.*	Weight after fast	Pentose		
			Liver	Kidney	Muscle
		gm.	mg.	mg.	mg.
Fasted control rats	160	163	56	34	126
	162	163	65	50	88
	164	149	58	49	99
	166	156	56	46	125
	172	170	51	50	81
	175	167	61	66	90
	214	162	40	37	92
	219	171	56	45	81
	168	165	41	71	86
	169	178	54	42	88
	170	164	43	44	49
	171	158	48	68	88
Average.....			53	50	91
Xylose-fed rats	161	154	83	94	105
	163	167	88	107	95
	165	144	86	116	113
	167	150	105	121	133
	173	170	73	83	83
	176	175	92	128	93
	215	162	83	103	88
	218	148	99	126	89
	177	189	95	134	76
	178	177	93	120	81
	179	205	88	120	88
	180	182	81	109	62
Average.....			89	113	92

\* The determinations with the first eight rats in each group were made in pairs, a fasting control experiment being carried out at the same time as a xylose feeding experiment. Thus the experiments with Rats 160 and 161, with Rats 162 and 163, etc. represent paired experiments.

It has been maintained by some workers that the administration of pentoses results in a mobilization of glucose. Blanco (10), in a study of the glucose content of the blood by the method of Somogyi, noted definite increases in the glucose of the blood 3 to 3.5 hours after oral or intravenous administration of xylose; the results after subcutaneous injection were variable. We have determined total reducing substances in trichloroacetic acid filtrates of the blood by the method of Hagedorn and Jensen and

TABLE II

*"Pentose" Content of Tissues of Rats after a 24 Hour Fast and after Glucose Feeding*

All results are calculated as mg. of xylose per 100 gm. of tissue.

	Rat No.*	Weight after fast	Pentose			
			Liver	Kidney	Muscle	Blood
		gm.	mg.	mg.	mg.	mg.
Fasted control rats	239	137	45	56	91	11
	240	148	50	41	55	7
	242	156	50	50	71	7
	244	156	54	59	63	6
Average.....			50	51	70	8
Rats fed glu- cose	238	134	46	49	80	11
	241	157	48	47	54	Lost
	243	155	49	40	74	8
	245	154	46	57	65	7
Average.....			47	48	68	9

\* The procedure of each feeding experiment was checked by a fasting control experiment carried out simultaneously. Thus the experiments with Rats 239 and 238, with Rats 240 and 241, etc. are paired experiments.

have calculated the values obtained in terms of xylose. We have also estimated the xylose content (pentose) of these blood filtrates by the McCance method already described. The figures obtained with fasting control animals and with animals killed 3 hours after xylose feeding are presented in Table III. By subtracting the values for xylose from the total reducing substances, we have obtained approximate values for total reducing substances not xylose.

The pentose (xylose) content of the blood as determined by the

TABLE III

*Composition of Blood in Fasted Rats and in Rats after Feeding of Xylose*

All results are expressed as mg. per 100 cc. of blood calculated as xylose.

	Rat No.	Group No.*	Total reducing substances (a)	Xylose (b)	Total reducing substances not xylose (a - b)
			mg.	mg.	mg.
Fasted control rats	220	1	125	9	116
	221	1	107	10	97
	225	2	122	7	115
	226	2	90	6	83
	227	2	115	8	106
	231	3	100	7	92
	232	3	116	7	109
	233	3	122	10	112
	234	3	140	7	132
	248	4	135	6	128
	249	4	106	8	99
	250	4	112	7	105
Average.....			116	7	108
Rats fed xylose	222	1	145	30	115
	223	1	145	30	115
	224	1	142	30	112
	228	2	187	39	148
	229	2	176	33	143
	230	2	172	40	132
	235	3	175	32	142
	236	3	147	34	113
	237	3	190	34	156
	251	4	175	31	143
	252	4	150	30	119
	253	4	155	34	121
Average.....			163	33	130

\* The analyses of the blood of all rats (experimental and control) of the same group were carried out simultaneously.

method of McCance showed a considerable and remarkably uniform increase 3 hours after the xylose feeding. Corley (11) who fed *d*-xylose to rabbits in amounts ranging from 1 to 3 gm. per kilo, amounts smaller than those fed in our experiments, observed

increases in the unfermentable reducing substances of the blood, which were much more marked than those observed in the present study with rats.

The total reducing substances not xylose (Table III) showed a very slight tendency to increase after a 3 hour xylose absorption period. The results were too variable and the increases too slight in comparison with the fasting control values to be considered entirely significant. Further work more carefully controlled is necessary to determine whether these slightly increased values are significant or within the limits of error of our experimental procedure.

The experimental data obtained in this and the preceding paper are being extended. Further studies on the nutritive value and utilization of the pentoses are in progress in this laboratory.

#### SUMMARY

1. The method of McCance has been adapted to the determination of the pentose content of trichloroacetic acid filtrates of tissues and blood.

2. After oral administration of *d*-xylose to the white rat, the pentose contents of trichloroacetic acid extracts of liver, kidney, and blood were increased. Under the same experimental conditions no changes in the pentose values of trichloroacetic acid extracts of muscle were observed.

3. The oral administration of glucose did not influence the pentose content of the tissues studied.

4. The increase in the reducing substances of the blood, other than those which yielded furfural on treatment with hydrochloric acid (*e.g.* pentoses), after a 3 hour absorption period after xylose feeding, was so slight as to be almost within the range of variation of the values for the control animals.

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# THE EFFECT OF UREA ON GLUCOSE DETERMINATION BY THE FORMOSE REACTION

BY HARDY W. LARSON

*(From the Biochemical Laboratory of the Metropolitan Life Insurance  
Company, New York)*

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## INTRODUCTION

Kingsbury (1) working in this laboratory discovered the interesting fact that glucose had an accelerating effect on the condensation of formaldehyde to produce formose. This catalytic effect of glucose is proportional to the amount of glucose present, and Kingsbury made this a basis for a new colorimetric method for reducing sugar in urine. The reagent which he developed is very sensitive to small amounts of sugar, but he found that urea interfered with the determination, giving low sugar values. Because of the many interesting possibilities which this reagent seemed to offer, it was thought a further study of this formose reaction in the presence of urea might prove profitable. Results obtained show that it is the ammonia resulting from urea decomposition which causes the low sugar values.

Kingsbury employed a dye, 2,4-dinitro-1-naphthol-7-sulfonic acid, as the color reagent for his glucose determination. This dye is reduced by the formose present with a resulting change in color from orange to brown. In the determination of reducing sugar in urine, certain substances, such as urea and creatinine, tend to lower the sugar values obtained by this method. Kingsbury found that by diluting the urine ten times, these substances were usually reduced to non-effective concentrations.

In the present study, pure 0.005 and 0.01 per cent glucose solutions were used, and 0.3 per cent urea, which would correspond to a somewhat higher than average urea concentration in a 1:10 diluted urine. At this concentration of urea, the glucose values obtained are 10 per cent too low. It is obvious that in order



to apply this method successfully to blood and urine, the urea must either be removed, or its effects inhibited.

*Attempts to Remove Urea, and to Vary the Kingsbury Reagent*

There is no satisfactory precipitant for urea which could be used. The use of xanthidrol was dismissed as impractical. Oxidizing reagents such as hypobromite could not be used on account of the resulting sugar destruction. Crystalline urease was prepared according to Sumner (2), and a solution of these crystals used to decompose the urea. It was found that the decomposition products of urea exerted a far greater effect on the reagent than urea itself. This was especially true of ammonia. A mere trace of ammonia inhibited the formation of formose. This observation was confirmed in later experiments. Prolonged aeration was necessary to rid the solution of ammonia at such low concentration of alkali. This aeration only served to destroy glucose, and this line of attack was abandoned.

Efforts to alter the Kingsbury reagent to overcome the urea effect were unsuccessful. This reagent is very near the saturation point, and any attempt to change the concentration only destroys the proportionality. It is a relatively easy matter to lower the urea effect by the addition of small amounts of such compounds as amyl alcohol, resorcinol, and nitrites, but this is done at the expense of proportionality. The addition of salts, for the most part, exaggerates the urea effect. This is especially true of the so called buffer salts, phosphates, acetates, etc.

Because of the failure to overcome this urea effect, it was deemed advisable to study the possible reactions which might cause it. There are four possible reactants: formaldehyde, urea, the dye itself, and urea decomposition products, as suggested by the urease experiments.

Urea-formaldehyde condensation immediately suggests itself, but there is little evidence to support this, although there probably is some kind of reaction between the urea and formaldehyde, even at this dilution.

The dye, 2,4-dinitro-1-naphthol-7-sulfonic acid, has been used as a precipitant for urea by Kossel and Gross (3), and urea flavianates are well known. In all but saturated solutions, these flavianates are soluble, just as are the urea salts of rufanic acid, which are in every way analogous to the flavianates (4).

Much attention has been paid to Wöhler's transformation of ammonium cyanate into urea, but little has been done regarding the reverse reaction if carried on at 100° in dilute solution. With regard to the possible decomposition products reacting with the reagents, the urease experiments have shown that any ammonia interferes with the determination.

### *Effect of Heat on Urea*

When a 0.3 per cent urea solution is heated in a boiling water bath for 15 minutes, there is quite a perceptible odor of ammonia, and on the addition of silver nitrate a precipitate of silver cyanate is formed. Heating a 0.3 per cent urea solution for 15 minutes at 100° results in the decomposition of 5.3 per cent of the urea. Heating for 15 minutes at 90°, 2.18 per cent of the urea is decomposed, while at 80° there is only 0.64 per cent, and at 70°, merely a trace.

Glucose determinations in the presence of 0.3 per cent urea were consequently run at these temperatures. When heated at 70° for 15 minutes, little or no urea effect was noticeable. The color developed, however, was too light to read with any degree of accuracy. Increasing the amount of formaldehyde present in the reagent, and increasing the time of heating, did not result in greater depth of color. Heating at 80° for 15 minutes gave a much more satisfactory color and only a slightly increased urea effect over the 70° heating; namely, about 1.5 per cent low for glucose. Doubling the formaldehyde concentration resulted in a somewhat better color. The urea effect was raised to 5 per cent when the solution was heated at 90°.

### *Effect of Ammonia on the Kingsbury Reagent*

The above shows conclusively that the so called urea effect is not due to urea itself, but to one or more of its decomposition products. In order to determine which of these products caused the lowering of the glucose values obtained, 0.3 per cent urea solutions were decomposed by urease, and the ammonia completely expelled either by boiling or aerating. Amounts of this liquid, corresponding to 5, 10, and 15 mg. of decomposed urea were then added to the 0.2 mg. of glucose solution to be determined. The glucose values obtained were not affected, showing

that it is the ammonia resulting from the decomposition of urea which causes the lowering of the glucose values.

In order to test the effect of ammonia salts on this glucose determination, ammonium chloride was added in concentrations varying from 1 to 10 mg. The addition of each mg. of ammonium chloride raised the colorimetric reading by 2 mm. Inasmuch as this change was directly proportional to the amount of ammonia present, a new use for the Kingsbury reagent as a quantitative test for ammonia is suggested.

*Mechanism of Urea Decomposition When Heated in Presence of Dilute Alkali*

When urea is decomposed by water, the initial decomposition is one into ammonium cyanate, which in turn is hydrolyzed to form ammonium carbonate. It is possible that in the transformation of urea into ammonium cyanate, some is converted to ammonium carbamate, but the amount is insignificant in comparison to the amount of urea which is decomposed by way of ammonium cyanate.

The velocity of transformation to ammonium cyanate is much greater than the speed with which the cyanate is subsequently hydrolyzed.

Werner (5) heated urea and barium hydroxide at 100° and found that ammonia was given off after a few minutes heating, but that it required a considerably longer period before there was any evidence of barium carbonate formation. Werner found that on heating urea with sodium hydroxide, the reaction proceeded to form sodium cyanate and ammonia in the first phase, and the cyanate was in turn hydrolyzed to form ammonia and sodium bicarbonate. Heating 0.2 N urea and 0.2 N sodium hydroxide for 1 hour, he found that 22.1 per cent of the urea was decomposed, 17.7 per cent into the first phase and 4.4 per cent into the second phase. The velocity with which urea decomposes is regulated by this second stage. The more sodium cyanate formed, the slower becomes the rate of decomposition. In the later stages of the reaction, the speed with which the sodium cyanate is hydrolyzed is the dominating factor in the urea decomposition. Masson and Masson (6) found that the addition of relatively small amounts

of cyanate in comparison to the urea present lowered the velocity of decomposition.

In a study of the velocity constants for the reversibility of the transformation of ammonium cyanate to urea, it is seen that this change is much more rapid and complete than in the case of urea transformation. Walker and Hambly (7) in working with 0.1 N solutions at 100° determined the equilibrium point of the system. This point is attained when 95 per cent of the ammonium cyanate is converted to urea, and 5 per cent of the urea has been transformed to cyanate. The equilibrium constant for this reaction is  $K_{100^\circ} = 0.000457$ .

It is interesting to note the effect of alcohol on the decomposition of urea and ammonium cyanate. Alcohol has a marked retarding effect on the decomposition of urea, and at the same time it greatly accelerates the conversion of ammonium cyanate to urea. Burrows and Fawsitt (8) have calculated the velocity constants for urea decomposition in the presence of varying amounts of alcohol. These constants decrease rapidly as the percentage of alcohol used is increased. Walker and Kay (9) have likewise studied the rate of urea transformation from ammonium cyanate in the presence of alcohol. The velocity constants which they have determined show that alcohol has a tremendous accelerating effect on the formation of urea from ammonium cyanate.

In the attempt to correlate the above facts regarding urea decomposition with their possible use in overcoming the urea effect on the Kingsbury reagent, the following stand out: (1) Regardless of whether urea or ammonium cyanate decomposes, there is always some ammonia formed. (2) The addition of cyanate ion retards urea decomposition. (3) The addition of alcohol decreases the velocity of decomposition of urea, and at the same time accelerates the formation of ammonium cyanate to urea and hinders the hydrolysis of metallic cyanate. The Kingsbury reagent was prepared to contain 10 per cent ethyl alcohol and 0.5 per cent sodium cyanate. With such a reagent, the urea effect was decreased by 50 per cent, giving glucose values about 5 per cent too low. The complete elimination of the urea effect is not possible (except at low temperatures), as long as there is any possible ammonia formation.

From the above it is evident that this glucose determination

cannot be satisfactorily applied to urine. Nevertheless, because of the extreme sensitivity of the reagent toward minute amounts of glucose, this reagent should prove valuable in determinations in which urea and ammonia are known to be absent.

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# THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PHYSIOLOGICAL RESPONSE

## III. FACTORS INFLUENCING THE EXCRETION OF URIC ACID\*

By ARMAND J. QUICK

WITH THE TECHNICAL ASSISTANCE OF MARY A. COOPER

*(From the Department of Surgical Research, Cornell University Medical  
College, New York City)*

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In a recent study, the writer (1) found that the decrease in the excretion of uric acid which results from the ingestion of sodium benzoate, can be completely prevented by supplying excess glycine. Since this amino acid, moreover, stimulates the excretion of uric acid, it was concluded that the action of benzoic acid depends on a depletion of the body's available glycine, thereby removing a stimulus necessary for the normal and efficient elimination of uric acid. Further studies made it apparent that this simple explanation did not suffice. New data have been obtained which indicate that there is a relationship between the conjugating mechanisms and uric acid excretion. With the presentation of these new findings, an attempt has been made to correlate as far as possible the various known factors which influence the excretion of uric acid.

The experimental procedure was simple. The neutralized drug dissolved in water was taken by mouth. A light breakfast consisting of coffee and a cruller was eaten 1 hour prior to the test, unless the nature of the experiment required fasting. A comparison of the uric acid excretion with and without breakfast showed practically no difference. The author served as the subject in most of the experiments, especially in those in which the work of other investigators was repeated. A number of the more crucial experiments, however, were also carried out on other normal adults, in order to obviate the danger of individual metabolic idiosyncrasies. Uric acid was determined by the Benedict-Hitchcock (2) method; the other analytical procedures were the same as employed in the previous papers of this series.

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

TA

*Factors Influencing Excretion of Uric Acid*

Stimulating			Depressing		
Factor	Amount <i>gm.</i>	Author	Factor	Amount <i>gm.</i>	Author
Amino acids					
Glycine	10	Lewis, Dunn, and Doisy (3)	Lactic acid	12-14	Gibson and Doisy (4)
Alanine	12	" " "	Glycolic acid	10	" "
Aspartic acid	18	" " "	Acetoacetic "	10-20	Quick
Glutamic "	20	" " "	High fat diet (ketosis)		Harding, Allin, Eagles, and Van Wyck (6)
Glucose	300	" and Corley (5)	Fasting (ketosis)		Lennox (7)
Glycerol	50	" " "	Benzoic acid	3-9	Lewis and Karr (9) Swanson (10)
Pyruvic acid	16	Gibson and Doisy (4)	Phenylacetic acid	3	Quick
High protein diet		Folin, Berglund, and Derick (8)	Substituted benzoic acids	3	"
Sodium chloride	30	" "			
Salicylic acid		Extensive literature			
Cinchophen					
Neocinchophen					

All of the data were secured on hourly samples of urine. Previous workers as well as the writer have repeatedly observed that the rate of uric acid excretion is fairly constant during the first 4 to 5 hours of the morning. This period is therefore most suitable. The importance of short period collections cannot be emphasized too strongly. It is now fairly definitely established that the variations occurring in the uric acid excretion during a low purine régime are not due to any appreciable changes in production or destruction, but are dependent upon the excretory mechanism, which is apparently very sensitive to various stimuli. It can readily be seen that after a stimulatory factor has spent its activity, a compensatory decrease in the excretion may follow, and *vice versa*, when a depressor causing an accumulation of uric acid in the blood is removed, excess uric acid will be excreted. While these changes in the rate of excretion are readily demonstrated by short period urine collections, a 24 hour specimen may show nothing if the compensatory phase is completely within that period. Much of the older literature in which the data are based on 24 hour collections is therefore of negative value, and in some instances has even given rise to entirely erroneous conclusions.

#### DISCUSSION

The excretion of uric acid in normal subjects can be readily and markedly influenced by various factors. For the sake of conciseness, these are summarized in Table I.

On examining Table I, it immediately becomes apparent that a relationship exists between carbohydrate metabolism and uric acid excretion. The fact that Harding *et al.* (6) found a direct connection between the height of acetone excretion and the rise in blood uric acid suggests that either ketosis itself or the lack of an active antiketogenic carbohydrate derivative brings about a retention of uric acid. The increase in blood uric acid observed after a high fat diet, and in fasting, can thus be readily explained. It is probable that even the gradual decrease in uric acid output during short periods of fasting (Neuwirth (11)) can also be accounted for on this basis. The ingestion of acetoacetic acid also slightly decreases the excretion of uric acid. Acidosis is not a factor, since the ingestion of sodium acetoacetate actually increases the alkali reserve. Lennox (7) has, furthermore, found



that sodium bicarbonate does not relieve the retention of uric acid during fasting.

From a clinical point of view, it is interesting to note that Ricci (12) has recently found that blood uric acid in fasting diabetic patients was high in cases not treated with insulin, and normal in treated cases; and furthermore, that the elevation of blood uric acid following a high purine diet was more prolonged in diabetic than in normal subjects.

In considering the factors that cause an increase in uric acid elimination, one is inclined to theorize that an active antiketogenic carbohydrate derivative exerts a definite stimulatory action. Glucose, except in exceedingly large amounts, has no effect on the uric acid output. The action of glycerol is more pronounced; 50 gm. will cause a definite increase, but 10 gm. have no demonstrable effect. Pyruvic acid, in marked contrast, in amounts as small as 7 gm. will cause a relatively pronounced increase in uric acid excretion. The question naturally arises whether pyruvic acid itself is the active carbohydrate derivative responsible for the increased elimination of uric acid. While no definite answer can be given, it seems fairly certain that pyruvic acid must at least be closely related to this active derivative. Unfortunately, the action of dihydroxyacetone and of methylglyoxal was not determined. 30 units of insulin with a high carbohydrate intake had no immediate influence on the uric acid output.

Lactic acid, as well as glycolic acid, actually depresses the excretion of uric acid. The marked depression reported by Gibson and Doisy (4) was not obtained, however, in the present study. Significantly, lactic acid also depresses the production of glycuronic acid as measured by the excretion of glycuronic acid monobenzoate, whereas alanine and also insulin increase the production (unpublished results). These observations make it appear unlikely that lactic acid is a normal intermediary in the catabolism of glucose. Certainly, the difference in the actions of lactic acid and pyruvic acid on uric acid excretion make one doubt whether there is a ready interconversion of these two compounds. A study of the effect of strenuous exercise on uric acid excretion should, in view of the large production of lactic acid, yield valuable results.

The stimulatory effect of amino acids on uric acid excretion has been repeatedly observed; and it is reasonable to suppose that the

low blood uric acid recorded by Folin, Berglund, and Derick (8) in patients on a high protein and low purine diet is dependent upon the high amino acid intake. It should be noted that all of the amino acids thus far studied are glycogenetic. This leads one to wonder whether their action is really direct or whether it is through a carbohydrate derivative which they yield. In an earlier paper (13) the writer postulated that all glycogenetic amino acids probably yield a common derivative, which the organism can either utilize directly or can convert to glucose. On comparing the effects of approximately equivalent amounts of alanine and pyruvic acid, a striking similarity in their action on uric acid excretion can be seen. The same observation was made by Gibson and Doisy. Such results suggest that the stimulatory action may be due to the pyruvic acid derived from alanine rather than to the amino acid itself. If this assumption is correct, it must be concluded that alanine undergoes oxidative deamination.

While the action of glycine on uric acid elimination is not specific, it is, nevertheless, more effective than other amino acids. In the first place, glycine, like other glycogenetic amino acids, stimulates the output of uric acid; and in the second place, it prevents and counteracts the depressing action of benzoic acid and substituted benzoic acids on the excretion of uric acid.

The striking action of aromatic acids on the elimination of uric acid has received comparatively little attention. Lewis and Karr (9) were the first to observe that benzoic acid caused a marked drop in the excretion of uric acid. Their work was corroborated by Swanson (10), who found a rise in the blood uric acid concomitant with the decrease in excretion. He disagreed with the conclusion of Lewis and Karr that the decreased excretion is brought about by a conversion of uric acid to glycine, but concluded that the excretion of uric acid is probably slightly retarded whenever large amounts of hippuric acid are being eliminated. Swanson, furthermore, found that benzoic acid did not cause a retention of urea, and Lewis and Karr noted a normal excretion of creatinine. While these findings indicate that benzoic acid exerts a direct action on the excretion of uric acid rather than a general nephrotoxic effect resulting in a generalized nitrogen retention, it must be remembered that uric acid is eliminated with greater difficulty than the other nitrogenous constituents and in early nephritis may be the only one that is retained.

In the present study it was found that not only benzoic acid, but substituted benzoic acids and phenylacetic acid as well, produce a prompt, marked, and prolonged decrease in the excretion of uric acid, and furthermore, that the period of depression in general coincides with the time required by the organism to eliminate the particular drug. The action of benzoic acid is of relatively short duration, while that of *o*-toluic acid and other ortho-substituted benzoic acids, which the body eliminates with difficulty, is much more pronounced. It will be recalled that in a previous paper of this series (14) it was found that substitution of benzoic acid in the ortho position greatly inhibits the conjugation with glycine. Phenylacetic acid, which is also excreted much more slowly than benzoic acid, likewise exerts a more prolonged depressing action on uric acid excretion. While the effect of 3 gm. of benzoic acid lasts only 2 hours, that of a similar amount of phenylacetic acid exceeds 5 hours.

It is significant that the depressing action of these aromatic acids completely abolishes the stimulatory effect of glycerol, pyruvic acid, and the various amino acids, with one exception. A small excess of glycine will counteract the action of benzoic acid completely, and that of *o*-toluic acid partially, but it is ineffective against phenylacetic acid (Tables II and III). It will be recalled that phenylacetic acid in the human is not conjugated with glycine but with glutamine.

While the stimulatory action of both pyruvic acid and alanine is entirely abolished by phenylacetic acid, it should be noted that the period of uric acid retention is shortened by either compound to approximately one-half. Preliminary studies on the rate of excretion of conjugated phenylacetic acid, moreover, show a definite increase when either alanine or pyruvic acid is fed. This leads one to conclude that pyruvic acid can furnish the precursor for glutamine. It is rather interesting that glutamic acid is ineffective.

While the foregoing results show a definite relationship between uric acid excretion and the conjugative mechanisms, it is not possible at present to offer any explanation. A further study of the action of other aromatic acids is desirable, especially from a clinical point of view. Such conditions as gout and eclampsia, in which there is a specific retention of uric acid, may perhaps have as their etiological factor a toxin similar to these common aromatic

TABLE II  
*Various Factors Influencing Excretion of Uric Acid*

Uric acid excretion						Subject	Summary of experiment
1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.		
mg.	mg.	mg.	mg.	mg.	mg.		
23.0	23.2	22.1	20.5	18.6		A	Fasting
23.0	27.0	24.3	23.0			"	"
24.0	23.0	21.3	25.0			"	Light breakfast
31.0	26.0	28.1	25.2			B	"
25.0	20.5	21.0	30.7			A	20 gm. acetoacetic acid. Fasting
30.0	26.6*	20.0				C	10 " " " "
24.0	18.4*	18.6	17.6			D	10 " " " "
29.0	26.7*	25.0	25.2			A	30 units of insulin. 100 gm. sucrose
20.1	17.4	17.6	20.0			"	10 gm. lactic acid
20.0	20.4	20.5	17.4			"	10 " " " Fasting
33.4	21.8*	21.6	21.0			B	15 " " " "
9.1	6.2	20.2	22.0			A	3 gm. benzoic acid
22.0	8.6	11.7	12.4	12.0	15.3	"	3 " phenylacetic acid
18.5	9.2	11.0	12.3			"	3 " " " and 3 gm. glycine
30.0	27.0	20.5	18.8			"	50 gm. glycerol
25.8	8.0	15.6				"	50 " " and 3 gm. benzoic acid
31.6	35.0	36.0	40.0			"	7 gm. pyruvic acid
12.0	7.2	10.9	21.2			"	7 " " " and 3 gm. phenylacetic acid
29.6	38.6	28.0	22.2			"	10 gm. alanine
14.3	7.7	10.7	22.0			"	10 " " and 3 gm. phenylacetic acid
22.5	28.0	20.4				"	8 gm. glutamic acid
24.2	11.5	14.5	20.2			"	8 " " " and 3 gm. benzoic acid
17.6	11.2	10.9	11.3			"	8 gm. glutamic acid and 3 gm. phenylacetic acid
25.8	27.2	27.2	24.0			"	7 gm. aspartic acid
23.0	11.6	24.0				"	7 " " " and 3 gm. benzoic acid

\* Drug taken at the beginning of the 2nd hour.

acids, or a damaged conjugative mechanism as a result of which the organism is deprived of its ability to handle the simple toxins arising from intestinal putrefaction and other common sources.

No attempt was made to investigate the effect of sodium chloride on uric acid excretion. Folin, Berglund, and Derick (8) stated that 30 gm. increased the output, but they gave no actual data. Harding, Allin, and Van Wyck (15) found that 15 gm. decreased the level of blood uric acid. This they attributed to an increased hydration of the blood. Further work, however, is necessary before one can arrive at any definite conclusion.

In view of the characteristic action of salicylic acid on uric acid excretion, it seemed desirable to make a comparative study of the three hydroxybenzoic acids, the conjugation of which has been

TABLE III

*Conjugation of Ortho-Substituted Benzoic Acids and Their Effect on Output of Uric Acid*

Time	o-Toluic acid				o-Chlorobenzoic acid 2.0 gm.			o-Methoxybenzoic acid 3.7 gm.		
	0.5 gm.	1.0 gm.	3.3 gm.*	3.3 gm. and 5 gm. glycine	Uric acid	Excretion		Uric acid	Excretion	
						Free	With glycine		Free†	With glycine
Uric acid excretion										
hrs.	mg.	mg.	mg.	mg.	mg.	gm.	gm.	mg.	gm.	gm.
1	24.0	26.0	10.8	19.8	12.0	0.20	0.13	15.8	0.05	0.05
2	23.0	15.7	8.3	17.8	11.0	0.24	0.27	14.1	0.06	0.06
3	21.0	14.5	8.5	14.0	17.0	0.22	0.18	9.5	0.02	0.06
4	25.0	17.0	13.6	15.5	19.0	0.13		25.0‡	0.06	0.21

\* The average excretion of o-toluic acid per hour was: free 0.01 gm., combined with glycuronic acid 0.21 gm., and combined with glycine 0.08 gm.

† Also combined with glycuronic acid.

‡ 3 gm. of glycine were taken at the end of the 3rd hour.

reported in the preceding paper of this series (16). Hanzlik's (17) excellent review of the literature on salicylic acid makes it unnecessary to discuss the individual papers. Since nearly all of the workers drew their conclusions from analytical results obtained on 24 hour urine specimens, it was felt that a study made on the hourly excretion should disclose the transient and finer variations which hitherto have been overlooked.

A comparison of the action of the three hydroxybenzoic acids on the output of uric acid discloses striking differences (Table IV). Salicylic acid promptly and markedly increases the excretion of

uric acid. A similar but much less marked effect is produced by *p*-hydroxybenzoic acid, whereas *m*-hydroxybenzoic acid, like benzoic acid, distinctly depresses the excretion. It should be mentioned that Denis (18) found that *p*-hydroxybenzoic acid did not affect the excretion of uric acid, and Rockwood (19) likewise concluded that neither the para nor the meta compound had any effect, although his data suggested a slight suppression by the latter acid. Both studies were carried out on 24 hour collections of urine. In attempting to correlate the action of these acids with

TABLE IV

*Effect of Hydroxy- and Methoxybenzoic Acids\* on Excretion of Uric Acid*

Time	<i>p</i> -Hydroxybenzoic acid				<i>m</i> -Hydroxybenzoic acid				<i>o</i> -Hydroxybenzoic acid			
	Urine vol- ume	Excretion of			Urine vol- ume	Excretion of			Urine vol- ume	Excretion of		
		Uric acid	Sodium chloride			Uric acid	Sodium chloride			Uric acid	Sodium chloride	
hrs.	cc.	mg.	gm.	per cent	cc.	mg.	gm.	per cent	cc.	mg.	gm.	per cent
1	33	30.0	0.32	0.96	30	11.1	0.32	1.12	22	35.0	0.22	1.00
2	57	32.0	0.55	0.96	45	7.5	0.47	1.04	35	58.0	0.34	0.96
3	37	21.8	0.46	1.24	35	9.7	0.42	1.20	49	46.0	0.49	1.00
4	33	22.5	0.51	1.56	40	16.3	0.54	1.36	55	44.0	0.59	1.08
	<i>p</i> -Methoxybenzoic acid				<i>m</i> -Methoxybenzoic acid				<i>o</i> -Methoxybenzoic acid			
1	33	23.0			34	12.6			26	16.0		
2	43	22.6			46	9.5			31	14.0		
3	35	20.0			51	10.4			18	9.5		
4	28	20.0			55	22.0			43	25.2†		

\* 3.5 gm. of the hydroxybenzoic acid, and 3.7 gm. of the methoxybenzoic acid were taken.

† 3 gm. of glycine were taken at the end of the 3rd hour.

their conjugation, it will be recalled that salicylic acid is excreted very slowly and entirely uncombined, *p*-hydroxybenzoic acid is conjugated partly with glycine and partly excreted uncombined, while *m*-hydroxybenzoic acid is combined mainly with glycine. The pharmacological action appears to depend on the presence of a free carboxy and hydroxy group in the molecule. One must bear in mind, however, that the metabolism of the glycuronic acid associated with these groups may be one of the important underlying factors upon which the activity of these compounds depends.

By converting the hydroxy radical to the inactive methoxy group, the stimulatory action is abolished. In the case of the ortho compound a marked depressing action on uric acid excretion is actually produced.

TABLE V  
*Action of Salicylic Acid on Uric Acid Output*

Salicylic acid taken.....	0.5 gm.	1.0 gm.	1 gm., 3 gm. benzoic acid
Time	Uric acid excretion		
hrs.	mg.	mg.	mg.
1	21.4	27.4	21.4
2	23.2	43.0	28.0
3	40.0*	37.0	18.5
4	58.0	37.0	36.0

\* 1.5 gm. of additional salicylic acid were taken at the end of the 2nd hour.

TABLE VI  
*Action of Cinchophen and Neocinchophen on Output of Uric Acid*

Time	Cinchophen, 1.0 gm.			Neocinchophen, 1.2 gm.		
	Urine volume	$\alpha_D^*$	Uric acid	Urine volume	$\alpha_D^*$	Uric acid
hrs.	cc.	degrees	mg.	cc.	degrees	mg.
1	24	-0.20	30.0	21	-0.15	30.0
2	28	-0.30	53.6	25	-0.20	41.6
3	35	-0.30	59.6	23	-0.20	38.4
4	36	-0.35	61.5	22	-0.15	38.4
5	39	-0.35	44.5	22	-0.20	53.5
6	51	-0.30	41.8	29	-0.15	47.5
7	30	-0.40	28.6	34	-0.20	39.0
8	44	-0.32	32.6	16		33.6

\* The rotation was observed in a 1 dm. tube. A slight levorotation, such as was observed after taking neocinchophen, is found in normal urine.

The amount of salicylic acid necessary to produce its effect is surprisingly small. For a man weighing 50 kilos, 1 gm. caused nearly the maximum effect, while a 0.5 gm. dose produced no stimulation of uric acid excretion whatsoever (Table V). It is interesting to note that salicylic acid and benzoic acid mutually counteract the effects of each other.

The action of cinchophen (phenylcinchonic acid) and neocinchophen (ethyl ester of *p*-methylcinchonic acid) appears to be similar to that of salicylic acid as seen from the results recorded in Table VI. The structural similarity between salicylic acid and cinchophen has been pointed out in a previous paper (13). It will be recalled that the second aromatic ring, condensed as in naphthalene, produces the same effect on the  $\alpha$ -carboxyl group as ortho substitution. Consequently, conjugation with glycine is inhibited, and this probably explains the slow elimination of cinchophen. There is evidence that both cinchophen and neocinchophen are readily oxidized to a hydroxy compound. Dohrn (20), and Rotter (21) have isolated a hydroxycinchophen from the urine, and Fürth and Kuh (22) have obtained a dihydroxynecinchophen. It is probable that neither cinchophen nor neocinchophen becomes therapeutically active until it is oxidized to the hydroxy compound. This view has also been expressed by Rotter. It should be noted that after the ingestion of cinchophen, the urine becomes definitely levorotatory. The constancy of this rotation suggests that a levorotatory compound is eliminated at a slow and fixed rate. When cinchophen is fed to a dog, a very marked levorotation of the urine is found. While the nature of the compound has not been determined, one is inclined to consider it a conjugation product, since it is difficult to see how any change in the cinchophen molecule can give rise to an asymmetrical carbon atom. There is no evidence of a levorotatory compound being produced after feeding neocinchophen.

No definite explanation for the action of salicylic acid, cinchophen, and neocinchophen can be given, but it seems quite probable that the action of these three drugs is essentially the same. From the results in Tables IV and VI, it can be seen that the excretion of uric acid is entirely independent of the urine volume, in sharp contrast to the output of chlorides, which is almost directly dependent upon the volume. Whether the extremely small volume of urine occurring after the ingestion of neocinchophen is due to the drug, cannot be answered until further studies have been made.

#### SUMMARY

1. The relationship between carbohydrate metabolism and uric acid output is discussed, and the possibility that an active carbo-



hydrate derivative may be necessary for the normal excretion of uric acid is considered.

2. Benzoic acid, various substituted benzoic acids, and phenylacetic acid depress the excretion of uric acid, and the duration of their action coincides with the time required for their conjugation and elimination.

3. The stimulating action on uric acid elimination of glycerol, pyruvic acid, and the various amino acids is completely inhibited by these aromatic acids; nevertheless, both pyruvic acid and alanine greatly shorten the period of effectiveness of phenylacetic acid. The depressing action of benzoic acid and the various substituted benzoic acids is prevented, at least partially, by excess glycine.

4. The excretion of uric acid is markedly increased by salicylic acid, moderately by *p*-hydroxybenzoic acid, and depressed by *m*-hydroxybenzoic acid.

5. The replacement of the hydroxy group by a methoxy radical completely abolishes the stimulatory action of the *p*- and *o*-hydroxybenzoic acids, indicating that the pharmacological action of these compounds is dependent upon a free hydroxy and carboxy group in the molecule.

6. The action of cinchophen and neocinchophen on the excretion of uric acid appears to be similar to that of salicylic acid. After the ingestion of cinchophen, a levorotatory substance appears in the urine.

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## TRYPTOPHANE METABOLISM

### III. THE RATE OF ABSORPTION OF *l*- AND *dl*-TRYPTOPHANE AND TRYPTOPHANE DERIVATIVES FROM THE GASTRO-INTESTINAL TRACT OF THE RAT

BY CLARENCE P. BERG AND LYLE C. BAUGUESS

(From the Laboratory of Biochemistry, State University of Iowa,  
Iowa City)

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#### INTRODUCTION

In any extensive metabolism study in which the substance under investigation is administered *per os*, knowledge concerning its rate of absorption is important. With the advent of the technique devised by Cori (1925), a comparatively simple direct method of obtaining such information has been made available. Thus far, only a few amino acids have been studied from the absorption standpoint. Cori (1926-27) determined the rates of absorption of glycine and *dl*-alanine in the rat. Wilson and Lewis (1929) applied the Cori technique, with some modification, in ascertaining rates of absorption of glycine, *d*- and *dl*-alanine, and of the sodium salts of glycine, *dl*-alanine, *d*-glutamic acid (the monosodium salt), and *l*-leucine. Stearns and Lewis (1930) studied the rate of absorption of the sodium salt of cystine in the rabbit, as did Wilson (1930) and Sullivan and Hess (1931) in the rat. In view of the fact, established by the investigations cited, that the rates of absorption of the various amino acids differ widely, we were led to undertake similar studies on tryptophane and tryptophane derivatives. The present paper is an account of our investigation of the rates of absorption of the sodium salts of free and acetylated *l*- and *dl*-tryptophane and also the rates of absorption of the ethyl ester hydrochlorides of *l*- and *dl*-tryptophane from the gastrointestinal tract of the rat.

## EXPERIMENTAL

The compounds used were all prepared in this laboratory. Table I summarizes the essential points concerning their preparation and properties.

In determining the rates of absorption, the technique of Cori (1925) was used. Unless the quantity of compound in the small volume of fluid which can be given a rat at one time (not much

TABLE I  
*Methods of Preparation and Properties of Compounds Used in Absorption Studies*

Compound	Method of preparation essentially as directed by	Melting point (uncorrected)	$[\alpha]_D^{20}$	Nitrogen	
				Found	Calculated
		°C.		mg.	mg.
<i>l</i> -Tryptophane	Cox and King (1930)	277	-33.2° to -33.5° (0.5 per cent solution in water)	13.68	13.72
<i>dl</i> -Tryptophane	Berg and Potgieter (1931-32)	283	0.0°	13.70	13.72
Acetyl- <i>l</i> -tryptophane	du Vigneaud and Sealock (1932)	189	+29.0° (0.4 per cent solution in 0.5 N NaOH)	11.30	11.38
Acetyl- <i>dl</i> -tryptophane	Berg, Rose, and Marvel (1929-30)	205-206	0.0°	11.30	11.38
<i>l</i> -Tryptophane ethyl ester hydrochloride	Same	220-221	+18.4° (0.6 per cent solution in water)	10.49	10.43
<i>dl</i> -Tryptophane ethyl ester hydrochloride	" (except with <i>dl</i> -tryptophane)	227-228	0.0°	10.45	10.43

over 2 cc.) is sufficient to permit absorption to continue over a period of a few hours, the error involved in determining the rate of absorption becomes large. Because tryptophane and acetyl-tryptophane as such are only sparingly soluble in water, solution was effected by adding an equivalent amount of sodium hydroxide. The ethyl ester hydrochlorides were dissolved in water. All solutions were of known concentration and the quantities fed were

checked analytically. At the end of the absorption periods the intestinal contents were prepared for analysis according to the method described by Wilson and Lewis (1929). As a precaution against the possible destruction of tryptophane, all concentrations were carried out *in vacuo*. Recoveries of *l*- and *dl*-tryptophane were estimated by Van Slyke's amino nitrogen method (1913-14, 1915). Acetyl-*l*- and acetyl-*dl*-tryptophane recoveries were determined by an adaptation of the Folin and Ciocalteu (1927) method for tryptophane. Both methods were employed in estimating the *l*- and *dl*-tryptophane ethyl ester recoveries, but since

TABLE II

*Content of Amino N and Folin-Ciocalteu Reducing Material in Intestine of Rats Fasted 24 Hours*

Rat No. and sex	Weight after 24 hr. fast	Amino N	Reducing material (calculated as tyrosine)
	gm.	mg. per 100 gm.	mg. per 100 gm.
1, ♂	134	10.1	10.1
2, ♂	128	9.9	6.9
27, ♀	114	11.9	8.5
28, ♀	114	10.9	11.3
29, ♀	136	14.0	6.6
30, ♂	132	8.9	8.0
55, ♀	124	8.6	6.8
68, ♂	129	12.3	10.3
69, ♂	131	11.1	14.1
70, ♀	139	10.1	6.0
Average.....		10.78	8.86

the figures are in good agreement, only the amino nitrogen data are presented. Both methods were found to give accurate results in estimating known quantities of the compounds for which their use was intended. The satisfactory recovery of the compounds under experimental conditions was also established, as follows: The combined gastrointestinal washings of two rats were divided into two equal parts. To the one of these was added a known weight of the compound in question; no addition was made to the second. Both were subjected to the routine procedure of preparing the washings for analysis and estimating the compound

added. The difference found represented the compound recovered. In each instance recoveries estimated agreed well with the actual additions made.

The average amino nitrogen blank and the average Folin-Ciocalteu blank were established on the gastrointestinal washings of a series of ten control animals, previously fasted 24 hours, administered 2 cc. of water by stomach tube, and sacrificed 2 hours later. In each case, the washings were subjected to the Wilson and Lewis (1929) procedure for precipitating proteins and reducing the volume. The results are recorded in Table II. The average value of amino nitrogen found was 10.78 mg. per 100 gm. of body weight. Wilson and Lewis found this average to be 12.03 in the spring and 7.42 in the fall. Our experiments were carried out in April and May. The substances reducing the Folin-Ciocalteu phenol reagent were calculated as tyrosine, since tyrosine is the standard used in that method. When used as a blank in determining the residual acetyltryptophane or tryptophane ethyl ester hydrochloride, the tyrosine value was calculated in terms of the derivative.

As a check on the entire procedure, glycine was administered to three animals and the average rate of absorption at the 2 hour period determined. 52.8 mg. per 100 gm. of body weight per hour were found, a figure in good agreement with Cori's 50 mg. and the 52.5 mg. reported by Wilson and Lewis for the same period.

The experiments on *l*- and *dl*-tryptophane, administered as the sodium salts, are recorded in Table III. The average figures of 65.3, 61.9, and 61.4 mg. per 100 gm. of body weight per hour obtained on *l*-tryptophane during the 2, 3, and 4 hour periods, respectively, are somewhat higher than the corresponding values of 57.4, 56.0, and 60.3 on *dl*-tryptophane. Wilson and Lewis have found the following descending order of absorption coefficients for the amino acids which they fed as the sodium salts: glycine (62.8), *d*-glutamic acid (monosodium salt, 61.6), *dl*-alanine (46.3), *l*-leucine (42.1). The rate of absorption of *l*-cystine fed as the sodium salt has been determined as 30.5 by Wilson (1930) and as 53.3 by Sullivan and Hess (1931). The two latter reports are based on different colorimetric methods of estimation. Our finding for *l*-tryptophane (62.9) is the same as the value reported for glycine; the *dl*-tryptophane rate is exceeded by glycine and glutamic acid.

TABLE III

*Rate of Absorption of l- and dl-Tryptophane Fed As Sodium Salts*

	Rat No. and sex	Weight after 24 hr. fast	Absorp- tion time	Trypto- phane fed	Trypto- phane recovered	Rate of absorption	Average rate of absorption
		gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.	mg. per 100 gm. per hr.
<i>l</i> -Trypto- phane	3, ♂	116	2	255.5	119.4	68.1	65.3
	4, ♂	112	2	263.5	148.1	57.7	
	9, ♂	113	2	261.3	116.7	72.3	
	10, ♂	106	2	263.5	137.2	63.2	
	5, ♀	115	3	295.3	106.3	63.0	61.9
	6, ♀	111	3	257.0	50.5	68.8	
	11, ♂	106	3	281.0	103.9	59.0	
	12, ♀	105	3	293.6	123.0	56.9	
	7, ♂	112	4	297.0	88.2	52.2	61.4
	8, ♀	107	4	278.2	0.8	69.3	
	13, ♂	112	4	291.1	44.5	61.7	
	14, ♂	105	4	278.2	28.9	62.3	
Average of all experiments.....							62.9
<i>dl</i> -Trypto- phane	15, ♂	118	2	263.3	147.3	58.0	57.4
	16, ♀	112	2	276.8	152.2	62.3	
	21, ♀	110	2	282.1	176.9	52.6	
	22, ♀	113	2	274.3	161.2	56.6	
	17, ♀	118	3	263.3	106.3	52.3	56.0
	18, ♀	118	3	263.3	110.9	50.8	
	23, ♀	109	3	290.8	118.1	57.6	
	24, ♀	113	3	280.3	90.9	63.1	
	19, ♀	124	4	255.6	31.3	56.1	60.3
	20, ♀	117	4	270.7	36.4	58.6	
	25, ♀	108	4	293.5	1.4	73.0	
	26, ♀	116	4	273.2	60.2	53.3	
Average of all experiments.....							57.9

If the rates of absorption are calculated in terms of millimols absorbed per hour per 100 gm. of body weight, however, the order becomes: glycine (0.837), *dl*-alanine (0.520), *d*-glutamic acid



TABLE IV

*Rate of Absorption of Acetyl-L- and Acetyl-DL-Tryptophane Fed As Sodium Salts*

	Rat No. and sex	Weight after 24 hr. fast	Absorp- tion time	Deriva- tive fed	Deriva- tive re- covered	Rate of absorp- tion	Average rate of absorp- tion	Amino N found
		gm.	hrs.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm. per hr.	mg. per 100 gm. per hr.	mg. per 100 gm.
Acetyl-L-tryptophane	31, ♀	125	2	312.6	138.0	87.3		13.0
	32, ♀	130	2	301.8	122.0	89.9		10.8
	40, ♂	116	2	338.0	165.8	86.1		9.1
	41, ♂	110	2	356.9	167.4	94.8	89.5	11.0
	33, ♀	106	3	369.9	113.6	85.4		8.6
	34, ♀	106	3	369.9	87.7	94.1		9.6
	38, ♀	103	3	390.5	149.3	80.4		8.9
	39, ♀	102	3	393.8	141.4	84.1	86.0	9.8
	35, ♀	102	4	393.8	56.0	84.5		13.0
	36, ♀	108	4	372.7	47.7	81.3		8.5
	37, ♀	100	4	402.1	56.5	86.4		10.9
	42, ♂	105	4	382.8	66.6	79.1	82.8	13.2
Average of all experiments.....							86.1	10.53
Acetyl-DL-tryptophane	43, ♀	100	2	297.4	106.6	95.4		14.0
	44, ♀	104	2	286.0	101.8	92.1		6.4
	45, ♀	123	2	242.2	58.2	92.0		6.0
	52, ♂	108	2	275.4	104.7	85.4		10.6
	53, ♂	106	2	280.9	102.8	89.1		10.9
	51, ♀	120	2	248.0	58.4	94.8	91.5	8.6
	46, ♂	103	3	350.7	75.9	91.6		16.1
	47, ♂	106	3	341.0	99.6	80.5		9.0
	48, ♂	109	3	331.4	49.8	93.9		12.9
	49, ♀	110	3	319.5	30.2	96.4		15.0
	54, ♂	101	3	357.9	68.2	96.6		7.9
	50, ♀	101	3	357.9	75.2	94.2	92.2	12.0
Average of all experiments.....							91.8	10.78

(0.419), *L*-leucine (0.321), *L*-tryptophane (0.308), *DL*-tryptophane (0.284), *L*-cystine (Sullivan and Hess, 0.222; Wilson, 0.127).

The experimental findings on acetyl-*L*- and acetyl-*DL*-trypto-

phane appear in Table IV. As previously indicated, the recoveries in this series of experiments are based on the reduction of the phenol reagent used by Folin and Ciocalteu (1927) for the estimation of tryptophane. Inasmuch as only acetyltryptophane was fed, mercuric sulfate precipitation to differentiate between tryptophane and other amino acids was not necessary. Therefore, this rather tedious step was omitted. Some difference was found in rates of absorption of acetyl-*l*-tryptophane and acetyl-*dl*-tryptophane, the former averaging 89.5, 86.0, and 82.8 mg. per 100 gm. of body weight per hour at the end of the 2, 3, and 4 hour periods respectively; the latter, 91.5 and 92.2 for the 2 and 3 hour periods. These values are all considerably greater than those for the corresponding free amino acids. If the rates of absorption of acetyl-*l*-tryptophane and acetyl-*dl*-tryptophane are calculated on the basis of the tryptophane absorbed, the average values become 71.4 for the former and 76.1 for the latter, still appreciably greater than 62.9 found for *l*-tryptophane and 57.9 for *dl*-tryptophane. The differences are probably due, at least in part, to the more acid character of the acetyl derivatives. The estimations of amino nitrogen in the gastrointestinal washings were made in order to throw some light, if possible, on the question of enzymolysis of acetyltryptophane prior to absorption. Berg, Rose, and Marvel (1929-30) found only slight cleavage *in vitro* even after 12 hours digestion with pancreatic or intestinal enzyme preparations and were inclined toward the opinion that the derivative is absorbed mainly unhydrolyzed. Averages of the amino nitrogen content of the gastrointestinal tracts after acetyltryptophane feeding were 10.53 mg. per 100 gm. for the *l* series and 10.78 for the *dl* series, values in good agreement with the control average 10.78 (Table II). These data show that, if acetyltryptophane does undergo hydrolysis, the cleavage is at least a very slow process; since there is no accumulation of amino N, the tryptophane, if liberated, must undergo absorption as rapidly as formed. We are inclined to interpret the data as additional evidence that acetyltryptophane is absorbed mainly as such.

The average rates of absorption of the ethyl ester of *l*-tryptophane, fed as the hydrochloride (Table V), are 39.8, 36.8, and 34.5 mg. per 100 gm. of body weight per hour for the 2, 3, and 4 hour periods respectively; the corresponding *dl*-tryptophane deriva-

TABLE V

### Rate of Absorption of Ethyl Esters of L- and dl-Tryptophane Fed As Hydrochlorides

[illegible]

tive gave the slightly higher values of 47.9, 40.1, 38.7, and 34.9 for the  $\frac{1}{2}$ , 2, 3, and 4 hour periods respectively. In terms of tryptophane, the average rates of absorption for the ethyl esters of *l*- and *dl*-tryptophane become 32.5 and 35.5 respectively, appreciably lower than the rates for the free amino acid modifications. Wilson (1930) showed that cystine fed as the sodium salt was absorbed at the rate of 30.5 mg. per 100 gm. per hour; fed as the hydrochloride, however, the rate dropped to 9.6. In the latter case, cystine was partially precipitated in the upper part of the small intestine. Upon neutralization of the hydrochloride in the tryptophane ethyl ester solution, the ethyl ester precipitates. This may be the cause of its lowered rate of absorption, as the precipitation of the cystine seems to be in the former case. If tryptophane ethyl ester can be absorbed as such, the more acid character of the intestine produced by the hydrochloric acid in the compound should be an accelerating factor rather than a retarding one. The question of the absorption of the compound is complicated further by the probability that the ester undergoes enzymatic cleavage and is absorbed largely as free tryptophane and ethyl alcohol. Berg, Rose, and Marvel (1929-30) have found that the ethyl ester is readily hydrolyzed *in vitro* by enzyme preparations. There is some possibility that the more acid character of the intestine may have functioned to retard enzymolysis. Unfortunately, our method of analysis was not adequate to differentiate between the free and the esterified amino acid.

#### SUMMARY

The rates of absorption of the sodium salts of *l*- and *dl*-tryptophane and the corresponding acetyl derivatives, as well as the rates of absorption of the *l*- and *dl*-tryptophane ethyl ester hydrochlorides have been determined. The following descending order of rates of absorption was observed: acetyl-*dl*-tryptophane, acetyl-*l*-tryptophane, *l*-tryptophane, *dl*-tryptophane, *dl*-tryptophane ethyl ester, and *l*-tryptophane ethyl ester.

There was no evidence of enzymatic cleavage of the acetyl derivatives of tryptophane prior to absorption.

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## THE BEHAVIOR OF CHOLESTEROL AND OTHER BILE CONSTITUENTS IN SOLUTIONS OF BILE SALTS

BY G. O. SPANNER AND L. BAUMAN

(From the Departments of Surgery and Medicine, Presbyterian Hospital, Columbia University, New York)

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Since cholesterol is the chief constituent of human gallstones, it seemed advisable to study its behavior in watery solutions of other bile constituents. By this means it was hoped to obtain information that would help to explain the crystallization of cholesterol that frequently occurs in human gallbladders and leads to the formation of gallstones.

Cholesterol, though insoluble in water, may attain a concentration of 1 per cent in human gallbladder bile. The latter is not always saturated with this sterol for some specimens may dissolve an additional 30 per cent of their cholesterol content if shaken with the finely powdered substance (1).

It has long been known that bile salt solutions have a solvent effect on cholesterol. Wieland and Sorge (2) showed that the sodium salt of desoxycholic acid was the most potent in this respect. This salt is present in human bile though probably combined with glycocoll or taurine in a concentration about one-third of that of the corresponding salts of cholic acid.

### *Solubility of Cholesterol in Solutions of Other Bile Constituents*

*Bile Salts*—A 0.2 per cent colloidal cholesterol solution was prepared according to Porges and Neubauer (3). The sodium salts of the chief acids that occur in human bile were used in 0.1 N (approximately 4 per cent) solutions. The turbidity of colloidal cholesterol disappears when it is added to the hot bile salt solution until a saturation point is reached. On cooling, the excess of cholesterol which crystallizes is removed by filtration. The cholesterol content of the clear filtrate was determined gravimetrically by the

Windaus procedure. For complete solution, 1 part of cholesterol requires about

80	parts of sodium cholate
80	" " " glycocholate
80	" " " taurocholate
50	" " " anthropodesoxycholate
20	" " " desoxycholate

*Sodium Oleate*—The solubility of cholesterol in sodium oleate solution is about the same as in the desoxycholate. However, such a solution soon becomes turbid unless sodium desoxycholate is added. A solution containing both salts in 0.1 N concentration will dissolve twice as much cholesterol as either one alone.

*Lecithin*—The turbidity of a 0.1 per cent colloidal lecithin suspension is removed by the addition of sodium desoxycholate. Such a mixture will dissolve twice as much cholesterol as the bile salt alone.

*Sodium Bilirubinate*—A solution of sodium bilirubinate in water has no solvent effect on cholesterol.

### *Dialysis Experiments*

*Cholesterol*—Colloidal cholesterol is non-dialyzable. A clear aqueous solution containing sodium desoxycholate (2.3 per cent) and cholesterol (0.1 per cent) was dialyzed into 400 ml. of water through a membrane prepared from 4 per cent collodion solution. In 4 hours 60 per cent of the bile salt and 40 per cent of the cholesterol had diffused through the membrane. Owing to the more rapid removal of the bile salt, part of the cholesterol separated as a flocculent precipitate in the collodion sac. The combination between bile salt and sterol is a loose one and therefore easily dissociated.

*Lecithin*—A solution containing lecithin (0.1 per cent) and sodium desoxycholate (1.3 per cent) was dialyzed into 100 ml. of water for 6 hours. The dialysate contained 0.018 per cent lecithin and 0.7 per cent of the bile salt. Both lecithin and cholesterol dissolved in desoxycholate diffused into a 10 per cent solidified gelatin.

*Sodium Oleate*—The rate of diffusion of sodium oleate is accelerated by the addition of sodium desoxycholate. 30 ml. of a solution

containing 4 per cent of the oleate and desoxycholate, respectively, were dialyzed into 300 ml. of water for 6 hours. 45.5 per cent of the oleate had passed the membrane as compared with 35.5 per cent without the bile salt.

*Bilirubin*—A 0.02 per cent solution of bilirubin in sodium desoxycholate dialyzes at the rate of 6 per cent in 7 hours.

*Human Bile*—A sample of human bile containing 10 per cent solids, 1 per cent cholic acid, and 0.1 per cent cholesterol was dialyzed for 8 hours. 0.2 per cent of the solids, 0.04 per cent of the cholic acid, and 0.001 per cent of the cholesterol had passed the membrane. Cholic acid was determined by the colorimetric method of Chiray and Cuny (4).

### Ultrafiltration

A solution containing 2.16 per cent of sodium desoxycholate and 0.089 per cent of cholesterol was filtered through a collodion membrane with the aid of suction. The filtrate contained 1.67 per cent of the bile salt and 0.047 per cent of the sterol.

### DISCUSSION

The experimental results favor the probability that solution of cholesterol in bile is maintained by the bile salts, soaps, and phosphatides. It is conceivable that a paucity of these substances in the bile secreted by the liver or their relatively more rapid absorption through the gallbladder wall may induce crystallization of cholesterol, and this may well be the first stage in gallstone formation. In cases of gallstones, cholesterol crystals are almost invariably present in the gallbladder bile. In cholesterosis or "strawberry gallbladder" there is a deposition of free and combined cholesterol in the wall of the gallbladder. This may also be explained by the relatively more rapid absorption of bile salts, thus permitting separation of cholesterol in the mucous membrane.

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1

2

3

## THE EFFECT OF EPINEPHRINE ON LIPID EXCRETION

BY ELSIE HILL AND ALFRED E. KOEHLER

(From the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara)

(Received for publication, July 7, 1932)

That the lipid excreted in the feces is independent of ingested fat and persists even on a fat-free diet was demonstrated by Hill and Bloor (1). Work by Holmes and Kerr (2) has led them to similar conclusions as to the constancy of composition of the fecal fat and its independence of the food fat. Bloor (3) further noted that considerable fatty material was produced in a Thiry fistula in a dog. Sperry (4) showed that in dogs the lipid excretion may continue for as long as 5 weeks on fat-free diets. This author (5) also ruled out the possibility that the fecal lipids had their origin in the bile secretion by exclusion of the bile from the gastrointestinal tract by means of a bile fistula. A close similarity was found between the excreted lipids in normal and bile fistula animals. The possibilities remained that the fecal lipids were actually excreted by the intestinal epithelium cells or that they were derived from desquamated epithelial cells, bacteria, etc. Sperry (6) separated the bacterial fraction of feces by centrifuging and found a considerable portion of the total lipids in the cellular fraction. He concluded, "The results indicate that the fecal lipid excretion is composed of lipids contained in various cellular structures such as bacteria, desquamated epithelial cells, protozoa, etc., of the feces."

Various effects of epinephrine on blood fat have been noted. Alpern and Collazo (7), Raab (8), and Alpern and Tutkewitsch (9) showed that the blood fat may be decreased by epinephrine, while on the other hand Bornstein and Müller (10) noted no consistent changes. Fleisch (11) demonstrated a considerable increase in the blood fat 24 hours after the injection but not in the first few hours. Himwich and Spiers (12) using dogs reported a definite

increase in the blood fat although variable results were obtained in some experiments, depending apparently upon the epinephrine dosage.

The recent work of Long and Venning (13) showed, however, that epinephrine probably has no effect on blood fat. The Stewart and White (14) method and its modifications such as used by Himwich and Spiers is affected by carbohydrates due to the formation by the alkali of organic acids which are included in the titration. Long and Venning showed that if the fatty acids are separated after saponification by precipitation with HCl and then titrated no increase in plasma fat could be demonstrated after epinephrine administration.

In the experiments to be reported, the effect of epinephrine injections in the white rat on the excreted fat was studied.

### *Method*

Two or three groups of three rats each were placed in rat metabolism cages. The feces and urine were collected by means of a large funnel and the feces separated by means of a fine wire mesh across the lower portion of the funnel. The diet was as follows:

	Groups 1 to 3	Groups 4 and 5
	<i>parts</i>	<i>parts</i>
Casein.....	3	3
Starch.....	3	3
Agar.....	1	1
Salt mixture (15).....	0.25	0.25
Dried yeast.....		0.50

The fat content of the diet was purposely kept very low so that in case any of the food was spilled and adhered to the feces, the resultant contamination would be negligible. A sample of the food analyzed by the same method as reported for the feces (alkaline hydrolysis) yielded 0.2 per cent fat for the diet of Groups 1 to 3 and 0.5 per cent for Groups 4 and 5.

The rats were placed on this diet for a period ranging from 8 to 11 days before the control period was started.

The feces were collected each day and placed in 95 per cent alcohol. At the end of each period 10 gm. of potassium hydroxide

were added for each 100 cc. and the mixture heated on a hot plate at a low temperature under a reflux condenser and boiled for 5 hours. The mixture was then cooled, water was added, and the mixture heated over a boiling water bath until most of the alcohol had evaporated. As the alcohol evaporated, water was frequently added. The solution was acidified with hydrochloric acid and thoroughly extracted with ether. The ether was evaporated and the residue extracted with petroleum ether. Dry sodium sulfate was added to the petroleum ether, and after evaporating to a convenient volume the petroleum ether extract was filtered into weighed dishes, dried in a vacuum desiccator, and weighed.

In the experiments on Groups 4 and 5, the feces were dried to constant weight at 98°. The dried feces after weighing were saponified, and the lipids determined as described above.

### *Results*

The results are shown in Tables I and II. The rats in Group 1 were not full grown. In this group an ether extract of the acidified hydrolysate mixture was used after complete removal of the alcohol. Since non-lipid substances are taken into solution in the water dissolved in the ether, the ether extract was thoroughly dehydrated with anhydrous sodium sulfate. The advantage of an ether extract is the complete extraction of the hydroxy fatty acids and the disadvantage, the extraction of lactic acid and non-lipid resinous material. The epinephrine in Group 1, Table I, was given intraperitoneally three times daily. There was no glycosuria during the period of treatment. The feces for the first 24 hours of treatment showed only a slight increase (6.2 per cent) in the lipid excreted as compared with the control period, but on the 2nd and 3rd days the excretion increased by 73.0 and 48.2 per cent, respectively.

The food consumed, and consequently the ingested lipid, decreased considerably during the epinephrine administration. Although the lipid excretion increased on the 2nd day by 73 per cent, the ratio, lipid excreted to lipid ingested, increased from 1.14 to 4.02. After cessation of the epinephrine administration the lipid excretion returned to its original level.

Groups 2 and 3 received the epinephrine subcutaneously three times daily. In neither of these experiments was any reducing

TABLE I  
*Effect of Epinephrine on Lipid Excretion, Moist Feces Being Used for Analyses*

Group No.	No. of rats	Date	Weight	Food consumed		Lipid ingested		Lipid excreted		Lipid excreted per day	Change in excretion from control period	Lipid excreted Lipid ingested
				Total	Per day	Total	Per day	Ether extract	Petroleum ether extract			
1	6	1931	gm.	gm.	gm.	gm.	gm.	gm.	per cent			
		Aug. 26-30	860	276	55.2	0.552	0.110	0.6293	0.1259		1.14	
		" 31*	905	47.0	0.094	0.094	0.094	0.1336	0.1336	+ 6.2	1.42	
		Sept. 1* " 2*		27.0 42	0.054 0.084	0.054 0.084	0.054 0.084	0.2178 0.1865	0.2178 0.1865	+73.0 +48.2	4.02 2.22	
2	6	" 3- 6	885	194	48.5	0.388	0.097	0.4480	0.1120	-11.0	1.15	
		" 7- 9		152	50.6	0.304	0.101	0.4665	0.1555	+23.5	1.53	
		" 14-15	1146	154	77.0	0.308	0.154		0.0898	0.0449		0.291
		" 16† " 17† " 18†		40 62 70	40.0 62.0 70.0	0.080 0.124 0.140	0.08 0.124 0.140	0.08 0.124 0.140	0.0552 0.0990 0.0655	0.0552 0.0990 0.0655	+22.9 +120.5 +45.8	0.690 0.796 0.470
3	6	" 19-21	1094	220	73.0	0.440	0.143	0.2863	0.0954	+112.4	0.666	
		" 25-27	1411	172	57.3	0.348	0.116	0.120	0.040		0.345	
		" 28†	1409	52	52.0	0.104	0.104	0.0625	0.0625	+56.2	0.601	
		" 29† " 30†		60 68	60.0 68.0	0.120 0.136	0.120 0.136	0.0915 0.0975	0.0915 0.0975	+123.8 +143.7	0.764 0.714	
Average for Groups 2 and 3	12	Oct. 1- 2	1389	122	61.0	0.244	0.122	0.1580	0.0790	+97.5	0.646	
		Control					0.135		0.0424		0.325	
		During treatment					0.117		0.0785	+90.5	0.671	
		After					0.133		0.0872	+111.6	0.655	

\* 0.02 mg. of epinephrine per 100 gm. of body weight was given intraperitoneally three times daily.

† 0.02 mg. of epinephrine per 100 gm. of body weight was given subcutaneously three times daily.

TABLE II  
*Effect of Epinephrine on Lipid Excretion, Dried Feces Being Used for Analyses*

Group No.	No. of rats	Date	Weight	Food consumed		Lipid ingested		Weight of dry feces		Lipid excreted		Dry feces Food	Lipid excreted Lipid ingested	Lipid in dried feces	
				Total	Per day	Total	Per day	Total	Per day	Total	Per day				
4	9	1933		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.			per cent	
		Feb.	9-10	2036	241	120	1.2	0.60	38.2	19.1	0.3746	0.1873	0.16	0.31	1.02
		"	11-12		260	130	1.3	0.65	47.0	23.5	0.3210	0.1605	0.18	0.25	0.68
		"	13-14		216	108	1.1	0.54	39.5	19.8	0.3230	0.1615	0.18	0.30	0.82
Average.....															
5	9	Feb.	15*-16*	2048	164	82	0.8	0.41	28.4	14.2	0.4672	0.2336	0.17	0.57	1.64
		"	17-18		236	118	1.2	0.59	40.4	20.2	0.6416	0.3208	0.17	0.54	1.60
		"	19-20		274	137	1.4	0.69	51.0	25.5	0.4807	0.2404	0.19	0.35	0.94
		"	21-22	2123	274	137	1.4	0.69	46.7	23.4	0.4362	0.2431	0.17	0.35	1.05
		"	23-24		255	128	1.3	0.64	48.4	24.2	0.3523	0.1762	0.18	0.27	0.73
		Mar.	9-10	1601	245	123	1.2	0.60	42.4	21.2	0.3345	0.1673	0.17	0.28	0.79
Average.....	9	"	11-12		269	135	1.4	0.70	47.1	23.6	0.3545	0.1773	0.18	0.25	0.75
		"	13-14	1600	257	129	1.3	0.65	49.8	24.9	0.3860	0.1930	0.19	0.30	0.77
				257	129	1.3	0.65	46.4	23.2	0.3583	0.1792	0.18	0.28	0.77	
		Mar.	15*-16*		135	68	0.7	0.35	21.4	10.7	0.2925	0.1463	0.15	0.42	1.37
Average.....	9	"	17-18	1630	249	125	1.3	0.65	44.1	22.1	0.2967	0.1484	0.17	0.23	0.68
		"	19-20		252	126	1.3	0.65	52.0	26.0	0.3827	0.1914	0.21	0.29	0.74
		"	21-22		215	108	1.1	0.55	41.5	20.8	0.3283	0.1642	0.19	0.30	0.79
		"	23-24	1688	235	118	1.2	0.60	49.5	24.8	0.4082	0.2041	0.21	0.34	0.82

substance excreted in the urine. The lipid excretion increased at the maximum by 120 and 144 per cent respectively. In both of these groups the lipid excretion remained elevated for the 3 day period following the stopping of the epinephrine.

These experiments were repeated with groups of nine rats instead of six, and the dry weight of the feces was determined so as to find whether the changes in lipid excretion caused by the epinephrine had any relation to the variation in the amount of dry fecal material. The preliminary control analysis period was also extended so as to show the daily variation in excretion. The results are shown in Table II. In the results on the nine rats of Group 4, it is shown that the daily variations in the lipid excreted are relatively small. The only indigestible residue from the food ingested was that from the agar and the small amount from the dried yeast. The dried feces contained, in addition to the food residue, the bacterial and other cellular material plus the excretions from the intestinal tract. The ratio of dry feces to food ingested is therefore a constant as far as the residue of the food is concerned, but varies with changes in the cellular content of the feces. In other words, if the bacterial content of the feces increased there would then also be an increase in this ratio. In Group 4 this ratio was not altered by the epinephrine injections and remained constant throughout the experiment, although the lipid excreted the day after the epinephrine injection was nearly doubled. The percentage of the lipid in the dry feces was also nearly doubled by the epinephrine. During the period after the epinephrine injections, the total lipid excreted as well as its percentage in the dried feces returned nearly to normal.

The rats of Group 5 were the only ones that did not show an increase of lipid excretion after epinephrine administration, although the ratio of lipid excreted to lipid ingested increased from an average of 0.28 to 0.42. The fact that this ratio increased but the total lipid excretion remained unchanged may be related to the decreased food intake which was most marked in this group. The percentage of lipid in the dried feces was increased on the day of injection and then returned to the level of the control period.

#### DISCUSSION

In four groups of a total of twenty-seven rats, epinephrine in doses of 0.02 mg. per 100 gm. of body weight three times daily

caused an average increase of 101 per cent in the lipid excreted on the day following the injections. In one group (Group 5) of nine rats there was no significant change in the total lipid excreted.

The ratio, lipid excreted to lipid ingested, increased in all cases (252, 173, 122, 96, and 50 per cent) on the day of or the day following epinephrine administration as compared with the averages of the control periods. The interpretation of these ratios is complicated by the fact that there was a decreased food consumption in all cases (51, 48, 9, 31, and 47 per cent) which, if the lipid formation or secretion in the intestinal tract remained constant, would in itself increase the ratio. However, the average increase in the ratio, lipid excreted to lipid ingested, after epinephrine administration was 138 per cent while the decrease in the food intake was only 37 per cent.

In two groups of a total of eighteen rats the percentage of lipid in the dry feces increased by 95 and 78 per cent over the average of the control period.

In these two groups the ratio, dry feces to food ingested, varied relatively little during the control period and during and after epinephrine administration.

It is impossible at the present time to state the exact mechanism by means of which epinephrine may affect lipid excretion. The present experiments indicate that epinephrine probably increases the excretion of lipids from the intestinal wall, but there are many other possibilities to be considered such as increased lipid formation by bacteria, increased desquamation of intestinal epithelium, excretion of lipid through the bile and other digestive secretions, extrusion of lipid from the intestinal wall due to the pressor action of the epinephrine, changes in peristaltic action, changes in food intake, and digestion of the lipids and their assimilation.

#### SUMMARY

On a low fat diet it was found that the administration of epinephrine in subglycosuric amounts usually caused a definite rise in the fecal lipid excretion.

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## A METHOD FOR THE DETERMINATION OF NUCLEOTIDES IN BLOOD AND MUSCLE

By STANLEY E. KERR AND M. ELEANOR BLISH

*(From the Department of Biological Chemistry, American University of Beirut, Beirut, Syria)*

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In searching for the method best adapted to a study of the nucleotides of both muscle and blood under certain experimental conditions, none of the methods in use at the present time proved satisfactory to our needs. We were first attracted to the nephelometric method of Buell and Perkins (1928), but were unable to secure even approximately correct results on applying the method to pure solutions of adenine nucleotide.

In the method of Jackson (1923) the nucleotide is precipitated by uranium nitrate, extracted from the uranium precipitate by means of alkali, then ashed, and Nesslerized. No proof is offered that nucleotide is the only nitrogenous compound precipitated by uranium. Evidence is given in this paper that only about half of the nitrogen in the uranium precipitate is nucleotide, and the alkali extracts only a portion of the latter from the uranium precipitate. Moreover, about 40 per cent of the nucleotide is lost when the proteins of blood are precipitated by the Folin-Wu procedure.

Parnas (1929), like Buell and Perkins (1928), determines nucleotides by precipitation with uranium salts, followed by hydrolysis of the nucleotide and removal of uranium. After a second period of hydrolysis the liberated purine is precipitated by the Krüger-Schmidt procedure (1905), the copper is removed by means of hydrogen sulfide, and the purines are again precipitated, this time by the ammoniacal-silver method of Salkowski (1898). The ammonia is removed in a vacuum desiccator followed by boiling with magnesium oxide, and finally the purine nitrogen is determined by the Kjeldahl procedure. This method is obviously time-

consuming, and the accuracy of the Salkowski silver precipitation has been denied by Thannhauser and Czoniczer (1920).

The method we have developed is based partly on procedures selected from several of the older methods, with certain modifications and additions. As finally adopted the procedure consists of precipitation of the nucleotide by uranium acetate in the neutralized trichloroacetic acid filtrate of muscle or blood, hydrolysis of the nucleotide with acid, removal of uranium by means of sodium hydroxide, and precipitation of the purine by means of copper hydroxide. Purines are then separated from other nitrogenous compounds contained in the copper hydroxide precipitate by the copper bisulfite precipitation procedure of Krüger and Schmidt (1905), somewhat modified. Purine nitrogen is finally determined by the Kjeldahl method.<sup>1</sup>

#### *Reagents*

Trichloroacetic acid, 10 per cent.

Uranium acetate, saturated solution.

Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ,  $12\text{H}_2\text{O}$ ), 30 per cent.

Sodium hydroxide, ammonia-free, 4 per cent and 25 per cent solutions.

Acetic acid, glacial and 5 per cent.

Sulfuric acid (nitrogen-free), concentrated, and 10 per cent.

Copper sulfate, 20 per cent.

Sodium bisulfite, 40 per cent.

Hydrochloric acid, 25 per cent HCl gas.

Hydrogen peroxide, 30 per cent.

0.01 N HCl, made by diluting 0.1 N acid with  $\text{CO}_2$ -free water.

0.01 N NaOH, made by diluting 0.1 N alkali with  $\text{CO}_2$ -free water, and preserved in a paraffined bottle provided with a siphon and a soda-lime tube.

<sup>1</sup> In a previous study on the effect of contraction on the free purine and nucleoside content of frog muscle, use was made of part of the procedure outlined above (precipitation of purines by copper hydroxide and by copper bisulfite). This investigation, to be published shortly in Hoppe-Seyler's *Zeitschrift für physiologische Chemie*, was carried on by the senior author in collaboration with Professor Gustav Embden in the latter's laboratory in Frankfurt-on-the-Main. Since this publication on muscle purines may be somewhat delayed, it is necessary to note here that the details of the method published in this paper represent considerable additional experimentation.

The CO<sub>2</sub>-free water is prepared by bubbling CO<sub>2</sub>-free air through the water under a good vacuum.

*Tashiro's indicator* is prepared according to Tashiro (1920) by mixing solutions of methyl red and methylene blue in such a proportion that the resulting indicator gives a lavender or pink color in acid solution, changing to a green or yellowish green on addition of alkali. As Tashiro states, each investigator must determine the proportion himself, since methylene blue preparations vary greatly. The methyl red should be recrystallized from alcohol, then dissolved in 50 per cent redistilled alcohol to the point of saturation. The methylene blue is made up as a 0.25 per cent aqueous solution.

With our reagents 6 parts of the methyl red solution mixed with 1 part of the methylene blue (Grübler) gave the proper colors.

#### *Preparation of Sample*

Blood should be defibrinated and pipetted directly into 4 volumes of 10 per cent trichloroacetic acid, since, on standing, ammonia is liberated from the adenine nucleotide, and the resulting inosinic acid likewise breaks down to nucleoside or free purine (Mozolowski, 1929). After standing 20 minutes the mixture is filtered.

Muscle or other tissue must be brought as rapidly as possible to a state in which no deamination of the adenine nucleotide and further breakdown of the nucleotide takes place. We prefer to freeze the tissue in liquid air, then to powder it in a mortar chilled to such a low temperature by successive portions of liquid air that the latter no longer boils in the vessel. The powdered material is transferred to a previously weighed glass-stoppered Erlenmeyer flask containing a measured amount of 5 per cent trichloroacetic acid. After addition of the powdered tissue and mixing, the weight of the sample is obtained by a second weighing. Extra trichloroacetic acid is finally added to make a 1:10 dilution. The contents of the flask may be filtered after standing for an hour with occasional mixing.

The amount of filtrate to be selected for the determination should contain between 0.2 and 0.6 mg. of purine nitrogen. Amounts within this range would be contained in 0.5 to 1.0 gm. of muscle, or 5 cc. of blood for most species.

We have found it advisable to perform all analyses in triplicate

so that in case one sample meets with misfortune during centrifugation or distillation the results are still obtained in duplicate.

*Procedure for Determining Nucleotides*

Measure the trichloroacetic acid filtrate into a centrifuge tube with conical pointed tip. Add a drop of phenolphthalein solution and neutralize with 4 per cent sodium hydroxide. When a permanent pink color is obtained, add 5 per cent acetic acid until the pink color is just discharged. To each 10 cc. of blood filtrate add 1 cc. of saturated uranyl acetate solution and 2 drops of 30 per cent disodium phosphate solution. For muscle filtrates, use 1.5 cc. of uranyl acetate and 2 drops of phosphate per 10 cc. of sample. Mix with a stirring rod,<sup>2</sup> rinse off the rod, and allow the material to stand for half an hour or longer. If the supernatant liquid is not yellow, indicating no excess of uranium, add more uranium acetate, mix, and allow to stand another half hour. Centrifuge for 10 minutes to throw down the precipitated nucleotide and phosphate. If the sum of nucleoside and free purine is to be determined, decant the supernatant liquid into a clean centrifuge tube, otherwise discard.

Stir up the precipitate<sup>3</sup> with 1 cc. of 10 per cent sulfuric acid, rinse the rod with a few drops of water, cover the mouth of the tube with tin-foil, and immerse in boiling water for 45 minutes, thus hydrolyzing the nucleotide to free purine and ribose phosphate. After the hydrolysis, rinse down the sides of the tube, neutralize the acid to phenolphthalein with 4 per cent NaOH, render slightly acid with 5 per cent acetic acid, and centrifuge to throw down the precipitated uranium hydroxide and phosphate. If the solution remains alkaline, uranium is incompletely precipitated.

Decant the supernatant liquid quantitatively into a 30 or 50 cc. centrifuge tube with pointed tip, and retain for the determination of purine. Instead of washing the uranium precipitate, dis-

<sup>2</sup> It is advisable to have a separate rod for each tube so that the precipitate which clings to the rod may not be lost.

<sup>3</sup> The precipitate need not be washed if fresh blood or muscle is being analyzed. When working with specimens in which autolysis of the nucleotide has occurred, the uranium precipitate must be freed from adsorbed purines and nucleosides by dissolving in cold dilute sulfuric acid and reprecipitating by neutralization.

solve it in 1 cc. of 5 per cent sulfuric acid, rinse down the sides of the tube, and reprecipitate by neutralizing to phenolphthalein with 4 per cent NaOH, finally making slightly acid with acetic acid. Centrifuge and add the supernatant liquid to the tube containing the main portion of the purines.

To the combined purine solutions add 1 cc. of 20 per cent copper sulfate, then make the mixture faintly alkaline to phenolphthalein by adding 4 per cent NaOH drop by drop (about 1.3 cc.) with continual stirring. The end-point is noted by the change of color of the precipitated copper hydroxide from light green to deep blue. The supernatant liquid should be faintly pink. If not, adjust by addition of weak NaOH or acetic acid. After allowing to stand for half an hour centrifuge the material to throw down the copper hydroxide, which contains all the purines. Decant and discard the supernatant liquid.

Dissolve the precipitate in 1 cc. of 5 per cent sulfuric acid, neutralize carefully with 4 per cent NaOH until a little copper hydroxide remains undissolved, then add 1 drop of glacial acetic acid to bring the precipitate into solution. Now place the tubes in a beaker of boiling water; when thoroughly hot add 1 cc. of 40 per cent sodium bisulfite, stir, and continue the boiling for 3 to 5 minutes. A flocculent precipitate of cuprous oxide and the copper-purine complex should appear within a few seconds after addition of the bisulfite. Remove from the bath and rinse down the sides of the tubes with a 5 per cent solution of sodium sulfate from a wash bottle or pipette. The cuprous oxide has a tendency to float on the surface, but settles out if a little of the sodium sulfate solution is run into the center of the liquid. Centrifuge for 10 minutes. If any of the precipitate remains on the surface of the liquid, squirt a little sodium sulfate solution on it and again centrifuge. Decant and discard the supernatant fluid.

Dissolve as much of the precipitate as possible by stirring with 1 cc. of 25 per cent HCl and transfer to a 50 or 100 cc. Kjeldahl flask, carefully rinsing all traces of undissolved material into the flask. Add 1 cc. of concentrated sulfuric acid (nitrogen-free) and two solid glass beads or a boiling tube<sup>4</sup> and evaporate over a free

<sup>4</sup> Boiling tubes such as those described by Folin and Svedberg (1930) are more effective than beads in preventing bumping during the ashing and subsequent distillation.

flame until fumes appear. Allow 1 drop of 30 per cent hydrogen peroxide to fall directly into the hot liquid without touching the sides of the flask, then digest over a low flame for 10 minutes.

After cooling the mixture, add 15 cc. of ammonia-free water. The nitrogen content of the solution may now be determined by the usual Kjeldahl distillation procedure. We use a micro apparatus of Jena glass with standard ground-glass joints.<sup>5</sup>

#### *Determination of Nitrogen by Kjeldahl Distillation Method*

Cleanse the apparatus with steam by distilling over some water. Measure into a small receiving flask (a 50 cc. Erlenmeyer flask is convenient) 5 cc. of 0.01 N HCl and add 8 drops of Tashiro's indicator. To the flask containing the digested purines add 15 cc. of 25 per cent NaOH and distil for about 3 minutes with the condenser tube dipping into the 0.01 N acid, and another 3 minutes with the receiving flask lowered in order to rinse out the condenser tube. Rinse off the outside of the condenser with neutral CO<sub>2</sub>-free water, and finally titrate the distillate with 0.01 N NaOH to the end-point of Tashiro's indicator, the change from pink to green. A sharp end-point is obtained only when the acid, alkali, and wash water are CO<sub>2</sub>-free.

A blank must be determined for the reagents, especially since peroxide often contains acetanilide. This is best done by carrying the procedure through from the copper bisulfite stage to the end. 1 cc. of 20 per cent copper sulfate is neutralized and treated with bisulfite and the procedure continued as described above.

#### *Determination of Nucleoside Plus Free Purine*

The supernatant liquid separated from the uranium nucleotide precipitate may be analyzed for the sum of nucleoside and free purine as follows:

Hydrolyze<sup>6</sup> the nucleoside by adding sufficient sulfuric acid to

<sup>5</sup> These we obtain from Otto Kühner, Robert Mayer Strasse 18, Frankfurt-on-the-Main, Germany. Since the joints are of standard size the parts are replaceable. The flasks, either of 50 or 100 cc. size, have necks ground to fit the distillation apparatus.

<sup>6</sup> Much of the nucleoside will be lost when uranium is removed unless it is first hydrolyzed. In one experiment in which the hydrolysis was omitted, only 56 per cent of added adenosine was recovered, whereas 94 to 100 per cent was recovered when hydrolyzed (see Table IV).

make a 5 per cent solution and heating in boiling water for 45 minutes. Neutralize and remove the uranium as described under the determination of nucleotides. To the uranium-free solution add 1 cc. of 20 per cent copper sulfate for each 10 cc. of solution, and precipitate copper hydroxide as previously described. Continue the procedure exactly as described for nucleotides.

TABLE I

*Comparison of Nucleotide Nitrogen Found in Blood When Different Protein Precipitants Are Used*

Species	Protein precipitant	Nucleotide N	Authority
		mg. per 100 cc.	
Pig	Acetic acid	6.89*	Mozolowski (1929)
"	" "	8.88*	"
"	" "	7.67*	"
"	Tungstic acid	5.38*	"
"	{ Trichloroacetic acid	8.49	Kerr and Blish
"	{ Tungstic acid	5.18	
"	Trichloroacetic acid	10.84	" " "
"	" "	10.33	" " "
"	Tungstic acid	5.97†	Buell and Perkins (1928)
Man	Acetic "	3.97*	Mozolowski
"	" "	5.74*	"
"	Tungstic "	3.15*	"
"	{ Trichloroacetic acid	6.86	Kerr and Blish
"	{ Tungstic acid	3.81	
"	" "	4.1‡	Jackson (1923)
"	" "	4.3§	Erikson and Okey (1931)

\* Total purine N.

† Average of eleven samples, recalculated from Buell's data to nucleotide N.

‡ Average of eight samples.

§ Average of 190 determinations on sixteen women.

## DISCUSSION

### *Removal of Protein*

Jackson (1923) and Buell and Perkins (1928) removed the proteins from blood by the tungstic acid method of Folin and Wu. This procedure leads to large losses of nucleotide as may be seen by comparing the values obtained for nucleotide nitrogen by using different precipitants (see Table I). In two experiments we com-



pared the trichloroacetic acid and tungstate methods of removing protein, analyzing the same blood specimen, and found that the amount of nucleotide contained in the Folin-Wu filtrate was only 56 to 61 per cent of that found in the trichloroacetic acid filtrate. The acetic acid method of Parnas (1929) yields results intermediate between the tungstate and trichloroacetic acid values. By using our method with trichloroacetic acid, we found the recovery of adenine nucleotide added to blood to be 103.4 and 104.5 per cent respectively (see Table II), whereas, by the same method only 48.3 per cent was recovered when the Folin-Wu procedure was used to precipitate the protein. Buell and Perkins obtained 102 and 103

TABLE II  
*Recovery of Adenine Nucleotide Added to Pig Blood*

Ex- per- iment No.	Nucleotide N in blood	Nucleotide N added	Total	Found	Re- covery
	mg.	mg.	mg.	mg.	per cent
1	0.5152 } 0.5180 } 0.5166 in 5 cc.	0.221 to 5 cc.	0.738	0.7476 } 0.7476 }	104.5
2	0.3234 } 0.3290 } 0.3253 in 3 cc.	0.1326 to 3 cc.	0.4579	0.4592 } 0.4662 } 0.4620 }	103.4

per cent recovery of adenine nucleotide added to blood *filtrate*. Jackson likewise recovered, by his method, 99 per cent of the nucleotide added to a blood filtrate. Had these authors added the nucleotide to the blood *before* the protein precipitation, the loss would undoubtedly have been discovered.

#### *Precipitation of Nucleotide by Uranium Salts*

Jackson (1923), Buell and Perkins (1928), and Parnas (1929) each used the method of Thannhauser and Czoniczer (1920) for separating nucleotides from nucleosides and free purines, precipitating the nucleotide with uranium salts in presence of inorganic phosphate. We adopted the same procedure, but have observed that when free purines are present in considerable excess, as in

filtrates from autolyzed muscle, a portion of the purines is carried down in the uranium precipitate.

Jackson (1923) evidently based his method for determining nucleotides on the assumption that the uranium precipitate contains no nitrogen compounds other than nucleotide. He extracted the uranium precipitate once only with 4 per cent NaOH and determined the nitrogen content of the extract. We found that the uranium precipitate prepared from a trichloroacetic acid filtrate of pig blood contained 20.7 mg. of nitrogen per 100 cc. of blood, of which only 10.8 mg. was purine nitrogen. Analysis of a second specimen of pig blood, with trichloroacetic acid used as the protein precipitant, showed that although the uranium precipitated 16 mg. of nitrogen per 100 cc. of blood (of which 7.2 mg. was purine), only 11.7 mg. were extracted by two treatments with NaOH (Jackson extracted only once), and in this extract only 4.6 mg. represented purine. A third specimen of blood was studied in a similar manner. From the Folin-Wu filtrate uranium precipitated 14.4 mg. of nitrogen per 100 cc. of blood, of which 5.2 mg. were purine. A single treatment of the uranium precipitate by NaOH extracted 6.8 mg. of nitrogen. The purine nitrogen content of this blood as determined by our method, with trichloroacetic acid, was 8.5 mg.

Hence, it seems that in Jackson's method much of the nucleotide is lost in the precipitation of protein by the Folin-Wu method, only about half of the nitrogen in the uranium precipitate is nucleotide, and the extraction by alkali is not complete.

#### *Hydrolysis of Nucleotide and Removal of Uranium*

The nucleotide in the uranium precipitate is completely hydrolyzed to free purine and ribose phosphate by heating at 100° with 10 per cent sulfuric acid for 45 minutes. Heating in an autoclave for this period leads to a loss of purine amounting to 15 per cent.

Uranium is completely removed by means of dilute NaOH if the solution is brought to a reaction just acid to phenolphthalein.

#### *Precipitation of Purines*

Thannhauser and Czoniczer (1920) found that the precipitation of purines by the ammoniacal-silver procedure of Salkowski (1898) was incomplete, whereas precipitation by means of bisulfite in the

presence of copper as recommended by Krüger and Schmidt (1905) gave correct results. This procedure may be applied to the uranium-free filtrate provided the solution be transferred to small Kjeldahl flasks and the precipitation carried out while boiling. Since the precipitate and supernatant fluid must again be transferred to a centrifuge tube in order to separate the precipitate, we have found it simpler to concentrate the uranium-free purine solution in the centrifuge tube by adding copper sulfate and sodium hydroxide, and then to perform the Krüger-Schmidt purine precipitation in the same tube.

Salkowski (1879) first recommended the use of copper hydroxide to remove glucose from solution, with sodium hydroxide used to precipitate the copper. Van Slyke (1917) used calcium hydroxide to precipitate the copper, finding that it gave the optimum alkalinity for complete removal of glucose. Embden and his coworkers have repeatedly made use of this copper-lime method for the precipitation of phosphorus compounds of muscle, and also for nucleotides and purines (Embden and Wassermeyer, 1928). The presence of calcium, however, interferes with the subsequent precipitation of purines by the bisulfite, hence we employed NaOH to precipitate the copper. The purines are quantitatively carried down with the copper hydroxide when the reaction is made faintly alkaline to phenolphthalein. When this precipitate is centrifuged after half an hour and redissolved in 1 cc. of sulfuric acid, the result is simply a concentration of the purines to a small volume. The copper hydroxide contains also other nitrogenous substances which are not purines, since they are not precipitated by bisulfite.

Krüger and Schmidt (1905) recommended that purines be precipitated by first buffering the solution with sodium acetate, then adding sodium bisulfite, boiling, and finally adding copper sulfate. The addition of sodium acetate definitely retards or prevents the precipitation not only of the cuprous oxide which forms the bulk of the precipitate but also of the purines. Moreover, when sodium acetate is employed, basic copper salts are apt to separate. We have found that the precipitation of purines by bisulfite is complete in the absence of sodium acetate and even in a solution which contains mineral acid in small amount.

The procedure described in our method has been tested on pure

purine solutions and invariably yields close to theoretical results. The precipitation may be carried out in the centrifuge tube, heated by immersion in boiling water.

### *Determination of Nitrogen*

The determination of nitrogen in the copper precipitate offers no difficulties. The ashing is complete in 5 minutes if a drop of perhydrol is added after fumes appear, although we usually permit digestion to proceed for 10 minutes. The Kjeldahl distillation is equally simple when a micro apparatus with ground-glass connections is used.

### *Results of Analysis of Pure Solutions of Adenine Nucleotide*

When the above method was applied to pure solutions of adenylic acid and the calcium salt of adenosine triphosphate,<sup>7</sup> from 93.3 to 101.8 per cent was recovered, the average of fifteen determinations being 97.6 per cent (Table III).

When adenosine triphosphate (calcium salt) was added to pig blood before precipitation of the proteins, the recovery was 103.4 and 104.5 per cent in two experiments (Table II). The reason for the high results has not been determined.

Analyses of mixtures of adenylic acid and adenosine are recorded in Table IV. In these experiments the precipitated uranium nucleotide was washed with 0.4 per cent NaCl to remove adenosine.

In an effort to test our method on a biological fluid containing practically no nucleotide, we analyzed serum. Blood serum cannot contain more than 1 mg. of nucleotide nitrogen, since the amount of organic phosphorus present is less than 0.5 mg. per 100 cc. (Martland and Robison, 1926).

Analyzing dog serum, we found 0.42 mg. of nitrogen per 100 cc. by the method for nucleotides. This corresponds to 0.19 mg. of nucleotide phosphorus. If this nitrogen were not nucleotide but some other compound carried down with purine by the copper bisulfite precipitation, then the contaminating substance might introduce an error of 6 per cent on applying the method to human

<sup>7</sup> We wish to express our thanks to Professor Gustav Embden who kindly furnished us with these salts.

blood, provided this substance were equally distributed between serum and corpuscles.

The correctness of the method depends on the specificity of the

TABLE III  
*Determination of Adenine Nucleotide in Pure Solution*

Experiment No.	Nucleotide N in sample	N found	Recovery
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	0.221	0.216	97.8
2	0.213	0.208	97.5
3	0.213	0.201	94.2
4	0.213	0.210	98.4
5	0.426	0.402	94.2
6	0.426	0.398	93.3
7	0.221	0.225	101.8
8	0.221	0.223	100.9
9	0.221	0.225	101.8
10	0.332	0.319	96.1
11	0.332	0.319	96.1
12	0.332	0.324	97.6
13	0.442	0.437	98.8
14	0.442	0.427	96.8
15	0.442	0.437	98.8
Average.....			97.6

TABLE IV  
*Separation of Adenylic Acid and Adenosine*

Adenylic acid N in sample	Adenosine N in sample	Adenylic acid N found		Adenosine N found	
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
0.103	0.185	0.101	98.1	0.185	100
0.103	0.185	0.094	91.2	0.104	56*
None	0.370	0.008	2	0.347	93.8

\* In this experiment the adenosine was not hydrolyzed, with the result that nearly half the adenosine was lost when uranium was removed.

uranium precipitation for nucleotides and of the bisulfite precipitation for purines.

#### SUMMARY

A method is described for determining nucleotide nitrogen, based on the precipitation of nucleotides by uranium, hydrolysis

to free purine, removal of uranium, concentration of purine by means of copper hydroxide, separation of purine from other nitrogenous substances by a copper bisulfite precipitation, and Kjeldahl determination of the purine nitrogen.

Adenine nucleotide is recovered satisfactorily whether in pure solution, in mixtures with adenosine, or added to blood.

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## RICKETS IN RATS

### XIII. THE EFFECT OF VARIOUS LEVELS AND RATIOS OF CALCIUM TO PHOSPHORUS IN THE DIET UPON THE PRODUCTION OF RICKETS\*

By HELEN BENNETT BROWN,† ALFRED T. SHOHL,  
EDNA E. CHAPMAN, CATHARINE S. ROSE, AND  
ESTHER M. SAURWEIN

(From the Babies and Childrens Hospital and the Department of Pediatrics,  
School of Medicine, Western Reserve University, Cleveland)

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In the absence of vitamin D and ultra-violet light, an abnormal content of calcium and phosphorus in the diet causes rickets in rats. In our previous studies we have defined this salt defect primarily as an imbalance or disproportion between calcium and phosphorus. This may be expressed either in terms of the actual amounts consumed, or as a ratio of the calcium to phosphorus in the diet. In our previous communications, we have stressed the latter view-point.

Sherman and Pappenheimer (1) and collaborators (2) in their studies on rickets laid most weight on the actual phosphorus consumption and showed that small additions of phosphorus to the diet would prevent the development of rickets. This, of course, also alters the ratio. In the rickets-producing diet of McCollum and his collaborators (3) phosphorus is present in excess of the amount which prevents rickets in Sherman and Pappenheimer's study. McCollum *et al.* pointed out that their diet was relatively high in calcium, the ratio of Ca:P being 4.0.

Bethke, Steenbock, and Nelson (4) have investigated varying

\* Read before the American Society of Biological Chemists at Philadelphia, April, 1932.

† Part of the data offered are taken from the dissertation submitted by Helen Bennett Brown in partial fulfillment of the requirement for the degree of Doctor of Philosophy, Yale University, 1930.



proportions of Ca:P in the diet with and without cod liver oil and showed that the amount of cod liver oil necessary to prevent rickets depended upon the amount and proportion of the minerals in the diet. Bethke (5), with collaborators, has continued these studies, and reported them in Chicago in 1930, but to date has not published them, except in the annual report of the Ohio Agricultural Experiment Station. He has been kind enough to permit us to see all his data.

Kramer and Howland (6) have recently reemphasized that when rats are fed diets without vitamin D, the ratio of Ca:P of the diet is reflected in the content of calcium and inorganic phosphorus of the blood serum.

In our previous work we have used only high calcium and low phosphorus ricketogenic diets, such as McCollum's Diet 3143 (3) and Steenbock and Black's Ration 2965 (7). Change in the Ca:P ratio to 2.0 caused by the addition of phosphates causes significant differences towards normal (8). Schultzer (9) has obtained the same result with diminution of the calcium. However, since phosphate was added or calcium diminished, these studies also proved that *in this diet* there was a *relative* deficiency in phosphorus. It seemed, therefore, desirable for us to study anew the factors which determine the relative and absolute amounts of the calcium and phosphorus in the diet in relation to the production of rickets and bone formation.

### *Plan of Study*

Rats from the colony of the Institute of Pathology were weaned at 21 days and at 24 days put on the experimental diets. We have used the technique described by Goldblatt (10) of making the diet into a jelly with 3 per cent of gelatin in order to secure a more homogeneous distribution of the salts. Steenbock and Black's ration as modified by Hess and Sherman (11) was used. In this, corn-meal is substituted for corn. The alkalinity of the diet varied according to the calcium and phosphorus additions, but all diets were alkaline. The phosphorus content of the basal diet by analysis is about 0.113 per cent, or roughly half that found in Steenbock's Ration 2965. If the amount of calcium added to the diet is the same as for Ration 2965, 3 per cent of calcium carbonate (1.2 per cent of calcium), the ratio of Ca:P becomes 10:1 instead

TABLE I

*Effect of Varying Levels and Ratios of Calcium and Phosphorus of Diet upon x-Ray Pictures and Per Cent of Ash of Fat-Free Femurs, by Comparison of Ratios*

Composition of diet			x-Ray*	Ash of fat-free femurs†
Ca:P	Calcium	Phosphorus		
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>
1	0.12	0.12	++	41.8
	0.25	0.25	Normal	47.7
	0.50	0.50	"	51.7
	0.75	0.75	"	52.9
	1.00	1.00	"	48.7
	2.00	2.00	"	47.5
	3.00	3.00	"	46.1
	4.00	4.00	"	47.8
2	0.25	0.12	++	43.6
	0.50	0.25	Normal	48.1
	1.00	0.50	"	51.6
	2.00	1.00	"	52.8
	3.00	1.50	"	54.2
	4.00	2.00	"	51.7
3	0.37	0.12	+++	41.2
	0.75	0.25	+	45.3
	1.50	0.50	Normal	53.0
	3.00	1.00	"	54.7
	4.00	1.33	"	54.6
4	0.50	0.12	++++	41.4
	1.00	0.25	+++	44.0
	2.00	0.50	Normal	50.2
	3.00	0.75	"	52.6
	4.00	1.00	"	51.0
6	0.75	0.12	+++++	40.2
	1.50	0.25	+++++	42.0
	2.50	0.42	++	46.6
	3.00	0.50	Normal	51.1
	4.00	0.67	"	49.0
8	1.00	0.12	++++++	33.4
	2.00	0.25	+++++	38.0
	4.00	0.50	+	46.1

\* The number of plus signs indicates the degree of rickets.

† Average of individual determinations on three or more animals.

of 5:1. In this series of experiments we added varying amounts of  $\text{CaCO}_3$  and  $\text{Na}_2\text{HPO}_4$  to give the ratios and levels shown in Table I, so that we could observe the effect of various levels of calcium and phosphorus at the same ratio, or of the varying ratio of Ca:P at the same level of phosphorus or calcium.

After the rats had been fed these diets for 21 days, they were x-rayed and killed by bleeding. The blood serum was analyzed for calcium and phosphorus by the method of Kuttner and Cohen (12). The bones were in part saved for histological examination and in part for the determination of the ash content. The histological examination of the bone sections was made by Dr. Harry Goldblatt.

### Results

*Gain in Weight*—Of those animals which developed rickets some gained on the average only 1 to 2 gm. a week; some lost weight while rickets was developing. The presence of rickets in rats is therefore not necessarily associated with gain in weight.

*x-Rays*—The x-rays were graded independently of the knowledge of the diet, and the interpretation was checked independently by Dr. Goldblatt. Although these data cannot be regarded as quantitative, they were all examined and graded at one time and are comparative. The x-ray evidence is given in Table I and arranged according to ratios and in Table II according to levels of phosphorus. The x-ray evidence has been checked by histological examination by Dr. Goldblatt and found in agreement.

Table I shows that for the *same ratio* of Ca:P a diet becomes progressively *less rickets-producing* as the salt level is raised. Further, within the range used the greater the ratio at a given level of phosphorus, the more severe will be the degree of rickets which results. Other experiments have shown that if the calcium becomes still higher, 12:1, 24:1, or 36:1, a less severe degree of rickets results. Such animals do not gain weight or lose weight and may die early.

Table II shows that in those diets which contained more phosphorus than 0.5 per cent the production of rickets with high calcium diets is impossible. A diet with 1 gm. of phosphorus, Ca:P = 8.0, has 20 per cent  $\text{CaCO}_3$ , and the rats cannot eat a diet containing this amount of salt.

*Calcium and Inorganic Phosphorus of Blood Serum*—The analyses for the calcium of blood serum in these experiments ranged from about 10 to 11 mg. per cent in all cases. The values

TABLE II

*Effect of Varying Levels and Ratios of Calcium and Phosphorus of Diet upon x-Ray Pictures of Bones, by Comparison at Various Levels of Phosphorus*

Composition of diet		x-Ray*
Phosphorus <i>gm. per cent</i>	Ca:P	
0.12	1	++
	2	++
	3	+++
	4	++++
	6	+++++
	8	++++++
0.25	1	Normal
	2	"
	3	+
	4	+++
	6	++++
	8	++++
0.50	1	Normal
	2	"
	3	"
	4	"
	6	"
	8	+
1.00	1	Normal
	2	"
	3	"
	4	"
2.00	1	"
	2	"
3.00	1	"
4.00	1	"

\* The number of plus signs indicates the degree of rickets.

for the inorganic phosphorus are shown in Table III. The blood serum phosphate *decreases with increasing ratio at every level of calcium*. When one considers the data from the point of view of a

fixed ratio of Ca:P, the phosphate of the serum increases with the level of salts present. These data are in agreement with those of previous investigators (5, 6) and extend the experimental range.

*Bone Ash*—The values for the ash of the fat-free bone are given in Table I. The values clearly indicate that for a given level of calcium in the diet as the ratio of Ca:P is increased, the bone ash becomes progressively less. When the data are examined at a given ratio of Ca:P, it is evident that with increasing salt level the per cent of ash in the bones is increased. The single exception to this statement is found in the higher levels of diets with a ratio of Ca:P = 1.0. This probably should be classed as a high phos-

TABLE III

*Inorganic Phosphorus in Blood Serum of Rats Fed Diets of Varying Levels and Ratios of Calcium and Phosphorus*

The inorganic P is measured in mg. per cent.

Ca:P of diet	Ca in diet, per cent						
	0.106-0.125	0.25-0.375	0.50-0.75	1.0-1.5	2.0-2.5	3.0	4.0
	Inorganic P in blood serum						
1	4.8	8.0	7.9	8.0	7.7	10.3	
2		3.7	5.7	7.6	8.3	7.1	5.0
3		3.6	3.7	5.6		5.8	6.0
4			3.9	4.1	5.8	4.3	
6				3.6	4.0	3.6	5.4
8				2.5	2.9		3.0

phorus-low calcium diet, a study which is a separate problem. The data are in agreement with those of Bethke *et al.* (5).

#### DISCUSSION

The evidence furnished by blood serum phosphorus analyses, by x-ray and histological examination, and by ash content of the bones is all mutually confirmatory. The data are in agreement with the findings of other workers and show how various diets fit into a more general scheme. By an extension of the experimental range and a classification of the diets both by ratios of Ca:P and by salt levels it has been possible to point out a more fundamental relationship of the two elements in the diet. The acid-base factor

of the diet has been shown to be secondary in importance to that of the calcium and phosphorus (13). Inasmuch as the organic factors of the diet have been held constant, it is not proper to conclude that we have enumerated the only factors upon which the ricketogenic properties of the diet depend. However, diets as divergent as those of Sherman and Pappenheimer (Diet 84) and McCollum *et al.* (Diet 3143) and Steenbock and Black (Ration 2965) all are explained on this basis, without bringing into question the relative utilization of various forms of phosphorus (2). The addition of 70 mg. of phosphorus to 100 gm. of Diet 84, as in Sherman and Pappenheimer's Diet 85, which alters the ratio of Ca:P from 6.0 to 3.0, should prevent rickets, although the total phosphorus of 160 mg. is less than the 300 mg. of phosphorus in the ricketogenic Ration 2965 with a ratio of 4.0 or 5.0.

The diets of McCollum and associates (14), Lots 3407 and 3408, made with purified substances, and of Goldblatt's (15) modification of Korenchevsky's (16) diet in which casein and cooked egg white constitute the protein, and of Osborne, Mendel, and Park's (17) diet with edestin or lactalbumin as the protein, although of different content with regard to level and ratio of calcium and phosphorus, all fall in the zone that we have found produced rickets. With regard to the diets such as that of Korenchevsky where both the calcium and phosphorus are very low or in regard to the diets described by both McCollum and associates and Pappenheimer and associates in which the calcium is low and the phosphorus high, we desire to withhold comment, as this constitutes a separate problem. The production of rickets in other experimental animals or in children is likewise beyond the scope of this article. The main thesis is that for rats it is no longer adequate to describe a ricketogenic diet in regard to its calcium and phosphorus content unless the level and ratio are both stated.

#### SUMMARY

Analysis of the blood serum of rats for inorganic phosphate and examination of the bones by x-ray, histological sections, and ash analysis, after 21 days on a diet deficient in vitamin D, reveal that:

1. The ratio of Ca:P and the salt level are interdependent.
2. At a given level of calcium (or phosphorus), increasing the ratio of Ca:P intensifies the degree of rickets.

3. At a given ratio of Ca:P, increasing the level of salts diminishes the degree of rickets.

Hence, both the level and the ratio of calcium to phosphorus are necessary adequately to characterize the ricketogenic properties of a diet.

We desire to express our thanks to Dr. Harry Goldblatt of the Department of Pathology of Western Reserve University for his kind and generous assistance.

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## RICKETS IN RATS

### XIV. A DIET WHICH DEMONSTRATES THE EFFECT OF THE ACID-BASE CONTENT UPON THE PRODUCTION OF RICKETS AND ALSO CAUSES IDIOPATHIC TETANY

BY ALFRED T. SHOHL, HELEN BENNETT BROWN,  
EDNA E. CHAPMAN, CATHARINE S. ROSE, AND  
ESTHER M. SAURWEIN

(From the Babies and Childrens Hospital and the Department of Pediatrics,  
School of Medicine, Western Reserve University, Cleveland)

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Much that has been learned regarding rickets in experimental animals parallels the condition found in human infants. Fruitful as these studies have been, it has always been argued that rickets in rats differs from that in humans in several important particulars. Ricketic rats do not have idiopathic tetany in the course of healing rickets, and infants (1) may. In order to produce tetany in rats made ricketic by a high calcium-low phosphorus diet, it has been necessary to increase the phosphorus metabolized either by addition of phosphorus to the diet or by destruction of tissue through fasting.

With regard to the etiology of rickets much has been written concerning the importance of the acid-base factor in the diet, but the evidence is conflicting and does not carry conviction one way or the other. Rickets has been produced by McCollum *et al.* (2) when the calcium was present either as the chloride or the carbonate—hence with wide variations in the acid-base quality of the diet. Mellanby (3) also regards the acid-base factor as unimportant. Shelling (4) produced more severe rickets with acid than with alkaline diets. However, the acid diet contained slightly more calcium and less phosphorus than did the alkaline. György (5) has maintained that acidifying measures tend toward rickets and alkaline ones toward tetany. Zucker *et al.* (6) have stated that acid added to an alkaline diet to neutrality made it non-ricketogenic. McClendon (7) stated that alkali intensified rickets.



The high calcium-low phosphorus diet of McCollum as modified by Steenbock and Black has amply proved its utility and reliability in the production of experimental rickets. It has, however, the defect of its merits. It is so rickets-producing that rats cannot live long upon it unless given some source of vitamin D. Rats grow for about 3 weeks, then remain stationary in weight for 2 or 3 weeks and then decline. At the end of 10 weeks practically all have died. Certainly in this respect also such rickets fails to resemble the milder disease of infancy.

It was thought on the basis of observations made on human infants (1) that if this ricketogenic diet could be made less severe, a condition closer to the disease in children might be produced. If so, there would be a new opportunity to test the importance of the acid-base factor upon the etiology of rickets.

*Diet*—Ration 2965 of Steenbock and Black (8) has a Ca:P ratio of 4.0 to 5.0, depending upon the amount of phosphorus in the ingredients. The calcium content is approximately 1.2 gm. and the phosphorus 0.25 to 0.3 gm. per 100 gm. of diet. Hess *et al.* (9) suggested the use of corn-meal instead of corn and thus reduced the phosphorus content to about one-half (0.15 gm.) and increased the ratio of Ca:P to 10. At the original level of calcium this produces a very severe rickets. If the phosphorus level is twice that of the Steenbock Ration 2965 (0.5 gm.), it is very difficult to produce rickets even with very high calcium content. Unless the phosphorus is extremely low, a diet with a Ca:P of 2:1 produces practically normal animals (2, 10).

We therefore selected a diet which was moderately low in phosphorus and of the same Ca:P ratio as in the Steenbock ration. The diet contained yellow corn-meal 38, whole ground yellow corn 38, gluten 21, NaCl 1, and  $\text{CaCO}_3$  1.87. This gave a Ca:P of 4.0. By increase of the phosphorus this ratio was altered in small steps to 3.5, 3.0, 2.5, and 2.0. The last ratio, we had previously found, would produce normal animals.

The acidity of these diets was calculated by the method of Sherman and Gettler (11). The basal diet without  $\text{CaCO}_3$  was practically neutral (31 cc. of 0.1 N acid per 100 gm. of diet), but the addition of this salt produced an alkalinity of 344 cc. When 600 cc. of 0.1 N HCl, 300 cc. of 0.1 N HCl, or 151 cc. of 0.1 N NaOH were added the diet was made approximately 250 cc. acid, neutral, or

500 cc. alkaline. The basal diet was analyzed; the calcium was added as  $\text{CaCO}_3$  and the phosphate was added in the form of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ . The calcium-phosphorus content of the diet and the calculated acid-base value are given in Table I. Throughout the paper the values have been rounded off to 250 cc. of 0.1 N acid and 500 cc. of 0.1 N base, and the ratios given as 4.0, 3.5, 3.0, and 2.5.

*Methods*—Rats originally from Wistar Institute stock and bred in the laboratory of the Babies and Childrens Hospital were weaned at 21 days. The breeding stock was given the Sherman Diet B (12), consisting of wheat 66 per cent, dried whole milk 33 per cent,  $\text{NaCl}$  1.3 per cent, and in addition beef and liver, 5 gm. each once a week and lettuce twice a week. The young were con-

TABLE I  
*Acidity and Calcium-Phosphorus Content of Experimental Diets*  
The values are given per 100 gm. of diet.

Ca:P	Calcium	Phosphorus	Acid diet 0.1 N acid	Neutral diet 0.1 N base	Alkaline diet 0.1 N base
	gm.	gm.	cc.	cc.	cc.
4.15	0.760	0.183	256	44	495
3.57	0.760	0.210	268	34	485
3.09	0.760	0.246	278	22	473
2.59	0.760	0.293	286	15	465

tinued on the Sherman Diet B until 28 days old, when they were placed upon the experimental diet. They were continued upon this diet for 2, 3, or 4 weeks.

The reactions to galvanic stimulation were determined twice weekly by the method of Shohl and Bing (13).

The rats were x-rayed at weekly intervals. The diagnoses made by this method were subsequently checked by histological sections.<sup>1</sup> At the end of the experiment the rats were killed by bleeding under light ether anesthesia. The serum was analyzed for calcium and phosphorus by the method of Kuttner and Cohen (14). The protein was measured by the index of refraction. The pH and  $\text{CO}_2$  were determined by the method of Shohl (15). The

<sup>1</sup> For these observations we are greatly indebted to Dr. Harry Goldblatt of the Department of Pathology, Western Reserve University.

femurs were dissected and weighed fresh. They were then dried, extracted with alcohol and ether, and ashed. All the points for the 3 weeks experiments were made upon six animals. In the other groups there were three or four rats. All the above data were determined for individual rats, and the averages of the closely agreeing values are given in Tables I to IV.

*Progress*—The animals appeared normal at all times. None was seen in convulsions, though when we looked for it some seemed more excitable than normal and gave unduly large response to a clap of the hand or a sudden breath. There were no fatalities. Animals on Ration 2965 gained about 7 gm. per week. The experimental animals gained only 1 to 5 gm. per week. Al-

TABLE II

*x-Ray Diagnosis after 3 Weeks on Diets Varying in Acidity and Ratio of Ca:P*

Acid-base value*	Ca:P			
	4.0	3.5	3.0	2.5
Acid.....	++++†	++	+++	±
Neutral.....	++++	+++H.†	+++H.	—
Alkaline.....	++++	+++H.	++H.	±

\* See Table I and text.

† Plus signs indicate the degree of rickets.

‡ H. indicates healing.

though there were some exceptions the groups upon the acid and basic diets made poorer gains than those upon the neutral or alkaline diets.

*x-Ray*—The results for the x-ray pictures are given in Tables II and Fig. 1. Inasmuch as there was a series, and all the pictures were reviewed at one time, although the data are not quantitative, they are comparable. Those upon the diet at Ca:P = 4.0, whether acid, neutral or basic, showed moderately severe and developing rickets in all cases. The order of intensity was the same for all three groups. With a diet at Ca:P = 3.5 the rats upon the acid diet developed moderately severe rickets with no tendency to heal. Upon the neutral and basic diets the animals showed a severe condition but by 3 weeks considerable healing had taken place. Practically the same condition was seen with a diet at Ca:P = 3.0, but the rickets was not so marked. The

acid and basic groups were clearly differentiated, the former showing developing rickets and the latter healing rickets. Of the rats upon a diet with a  $\text{Ca:P} = 2.5$  only those upon the acid diet showed mild non-healing rickets; the others had never passed through what would be called a stage of rickets. At 2 weeks there was a slightly increased separation at the epiphyseal-diaphyseal junction, but at 3 and 4 weeks the bones were essentially normal in appearance in the x-ray pictures.

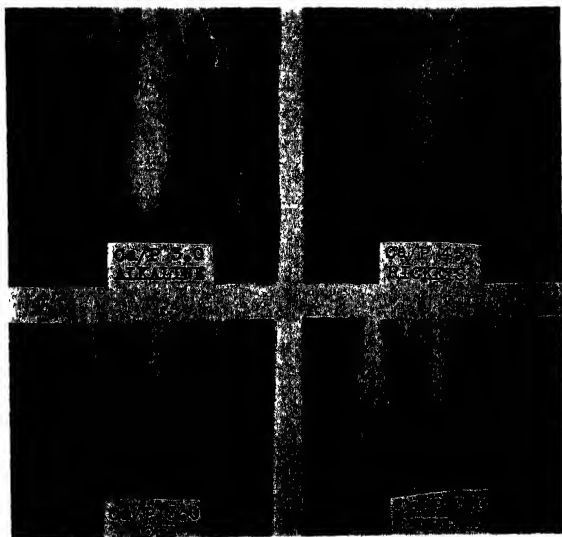


FIG. 1. x-Ray pictures of rats on various diets. (We are appreciative of the excellent services rendered by the Roentgenologic and photographic staff of the Babies and Childrens Hospital.)

Upon such a border-line regimen the degree of rickets is variable in different groups upon the same diet. For example, in one test, an acid diet at  $\text{Ca:P} = 3.0$  produced more severe rickets than one at  $\text{Ca:P} = 3.5$ . The diet at  $\text{Ca:P} = 3.5$  showed more healing in one test at 3 weeks than at 4 weeks. This phenomenon has often been observed in diets which produce a severe rickets, and it is not surprising to find a greater degree of variation in these marginal diets.

*Histological Examination*—The histological examinations were

made independently by Dr. Goldblatt. He reached practically the same conclusions as those found in the x-ray studies. He and Dr. Gerstenberger also independently examined the x-ray pictures and concurred in our diagnoses.

*Blood Serum Calcium and Phosphorus*—The results are given in Table III.

The group fed the diet with the Ca:P = 4.0 showed values in the blood serum of 10 mg. per cent or above for calcium, and inorganic phosphate depressed below 4.0 mg. per cent—values

TABLE III

*Calcium and Phosphorus in Blood Serum of Rats Fed Diets Varying in Acidity and Ratio of Ca:P*

The blood serum values are measured in mg. per cent.

Ca:P of diet.....		4.0		3.5		3.0		2.5	
Days on diet	Acid-base value*	Blood serum values							
		Ca	P	Ca	P	Ca	P	Ca	P
14	Acid	10.6	3.6	12.1	3.7	10.0	2.8	10.1	8.2
	Neutral	10.6	4.2	10.9	3.6	9.7	3.6	10.1	5.4
	Alkaline	11.3	3.6	12.2	3.4	9.9	5.2	10.7	5.5
21	Acid	12.3	3.7	11.5	4.2	11.0	3.0	8.8	7.4
	Neutral	13.3	3.1	7.5	6.6	8.7	3.9	11.0	6.7
	Alkaline	11.3	2.8	6.4	9.6	9.3	5.1	8.6	5.5
28	Acid	11.2	2.9	9.9	4.2	9.7	3.3	9.1	4.4
	Neutral	12.6	3.4	11.0	2.7	9.6	3.2	9.0	6.0
	Alkaline	10.7	2.4	10.1	2.2	12.9	2.8	11.1	2.9

\* See Table I and text.

typical of experimental rickets. The two intermediate groups at Ca:P = 3.5 and 3.0 gave low calcium and high phosphate values during the course of the experiment, returning subsequently to normal calcium and slightly elevated phosphate—data typical of tetany with recovery. The last group, at Ca:P = 2.5, produced serum calcium that was essentially normal and a phosphate above 5—data which have been interpreted as above the level of phosphorus found in rickets.

*Idiopathic Tetany*—Only one animal was found in tremor and none in frank convulsions. However, many low values of the

electrical reactions were encountered which were interpreted as latent tetany. Apparently these values were not constant, but a number of animals passed through a stage of increased neuromuscular irritability and returned to normal. If daily observations had been made, the duration and extent of this period could have been determined. Our main interest was to determine whether such a condition was present at all. Low electrical reactions were observed, principally in the group eating the diet with  $\text{Ca:P} = 3.5$ . Here it was encountered three times in each of the acid, neutral, and basic groups. At no other ratio was latent tetany observed in the acid groups. At a ratio of 3.0 hyperexcitability was observed three times in the neutral group and also in the alkaline group. Low reactions were encountered with the ratio of 4.0 and 2.5 only in the alkaline groups, three of each. As near as could be determined latent tetany occurred at a time when by x-ray evidence the rickets was not progressing but healing. Inasmuch as the tetany was induced by no known cause, it is proper to call it idiopathic tetany.

*Acid-Base Equilibrium of Blood Serum*—The serum was obtained without contact with air from the vena cava. That of the acid groups averaged 56 to 59 volumes per cent of  $\text{CO}_2$ , the neutral 58 to 61, and the alkaline groups 60 to 64. The pH in all groups was normal within our experimental error,  $\text{pH } 7.4 \pm 0.03$ . The acid-base changes were so slight that they can all be considered normal.

*Serum Proteins*—Serum proteins were determined and showed values of about 5 per cent with no significant variations in any groups. The values obtained by index of refraction are consistently lower than those previously reported by us determined by Kjeldahl digestion for normal rats and those with rickets and tetany. This method permitted the determination of proteins as well as other constituents on individual rats, and was used to detect differences and not the absolute amounts.

*Bone Ash*—The data for the ash of the last five bones are presented in Table IV. A 50 per cent deficient rat will show 16 to 20 per cent ash and rickets rats 25 to 40 per cent ash. In some cases the experimental groups in the range of 3.0 to 4.0  $\text{Ca:P}$  ratio fall between the values given above. This data with  $\text{Ca:P}$  and ash values which would be expected in a normal rat are

those fed the diet with Ca:P = 2.5 would be considered at the border-line of low normal values. The data for 2 and 4 weeks show that with a ratio of 4.0 the decrease in the bone ash is progressive. At a Ca:P = 2.5 the ash is higher and increasing. The ratio of Ca:P is a more important factor, therefore, than is the acid-base content of the diet. However, the acidity also shows its effect, for the rats upon the acid diets show a smaller ash content of the bones. This effect is small but significant. A similar effect of acid upon bone ash has long been known. Goto's (16)

TABLE IV

*Ash of Fat-Free Femurs of Rats Fed Diets Varying in Acidity and Ratio of Ca:P*

Days on diet	Acid-base value*	Ca:P of diet			
		4.0	3.5	3.0	2.5
		Ash of fat-free femurs			
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
14	Acid	40.7	42.5	41.4	45.8
	Neutral	37.1	40.1	47.6	46.7
	Alkaline	40.8	41.6	46.0	47.5
21	Acid	39.1	41.2	41.2	46.1
	Neutral	35.0	41.1	47.5	45.5
	Alkaline	38.0	44.6	49.0	48.7
28	Acid	36.7	34.4	40.9	45.5
	Neutral	31.0	34.8	34.7	51.2
	Alkaline	28.8	35.4	36.8	42.1

\* See Table I and text.

experiments upon rabbits showed conclusively that bone alkali can be reduced when animals are given acid.

#### DISCUSSION

In our experiments the conclusion is clearly warranted that acid diets tend toward the production of rickets. This finding is in agreement with the findings when phosphates of varying acidity were given to cure rickets (17). However, it is evident that the acid-base value is a secondary factor. The effect can only be shown in border-line diets when care is taken to keep other factors as constant as possible. Even then it might be argued that the

effect is due to the chloride and not to the acidity. No experiments have been reported to show the effect of chlorine upon the production of rickets. Likewise, with the diet made alkaline with NaOH, the effect might be due to the sodium. Therefore, sodium chloride was tested in amounts equivalent to the Cl present in the diets. The effect was to diminish the severity of the rickets rather than to intensify it. It cannot, however, be argued that the sodium was the cause of the tetany, for two reasons. Tetany occurred in the neutral and acid diets to which HCl and no sodium had been added; secondly, little tetany occurred in the diets with the largest and smallest Ca:P ratios, though the sodium was constant for all four diets.

That the alkali tends towards the production of tetany and acid or acid-producing substances towards its prevention or cure is well established. That alkalosis is not an essential feature of tetany in rats has been previously reported (18). In border-line conditions, such as detailed in this report, it seems probable that the alkaline or neutral diets favored the production of tetany. That it also was possible to produce tetany with acid diets is of interest. In one group of animals the calcium was depressed to 8.5 and the electrical reactions showed low or border-line reactions. Probably only a slightly greater amount of acid would be required to prevent the appearance of tetany in rats under these conditions. We selected the given acidities of the diets because they represent acid and alkaline diets at the extreme borders of normal diets.

The conclusion that we draw is that the ratio of Ca:P is the main factor and that the acidity (within the limits tested) is a distinct but secondary factor in the production of rickets.

For idiopathic tetany upon a ricketic basis the Ca:P ratio of the diet is of prime importance. Only when a mild rickets of the healing type is produced does tetany result. Gerstenberger and his associates (1) have shown that in ricketic human infants interrupted or insufficient therapy, such as to induce partial and incomplete healing, is associated with the development of idiopathic tetany. Our findings in rats parallel these observations. Rominger *et al.* (19) have maintained that tetany is associated with the third phase of rickets; namely, when the phosphorus retention is increased. Hence the phosphorus rise is the necessary condition for the calcium depression. We have previously shown that the phosphorus metabolism is essentially the limiting factor



in rickets in both rats and dogs, and that rickets is associated with a diminished but not negative phosphorus balance. Upon these diets in which the phosphorus values lie between low and normal it is not surprising to find that the phosphorus is deficient only for a variable period and that eventually enough is retained to meet the needs of the bones for the healing of the transitory rickets.

#### SUMMARY

A rickets-producing diet was altered stepwise by additions of phosphate until it became normal. Parallel diets were made acid, neutral, or basic.

Under such conditions in the intermediate groups, diets were found which produced the mild healing type of rickets when acid, and no rickets when neutral or alkaline.

The mild healing type of rickets was associated with idiopathic tetany.

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## THE ASSAY OF VITAMINS B AND G AS INFLUENCED BY COPROPHAGY\*

By N. B. GUERRANT AND R. ADAMS DUTCHER

*(From the Department of Agricultural and Biological Chemistry,  
Pennsylvania State College, State College)*

(Received for publication, July 19, 1932)

When autoclaved yeast is used as the source of vitamin G for supplementing a vitamin B complex-deficient diet, a lack of uniformity in the response of our experimental animals has often been noted. Some rats died within a period of 15 to 20 days without manifesting the characteristic symptoms of vitamin B deficiency until the time of death. Other animals grew at a slow rate for 6 to 9 weeks, at which time typical paralyses occurred and persisted for several days. In a number of cases we have been unable to obtain paralytic symptoms in a period of 20 to 24 weeks. These animals usually grew at a fairly uniform but subnormal rate.

The cause of these irregularities could not be traced to variations in initial weights of animals or to litter variations. It was found, however, that the difficulty could be eliminated almost completely if larger meshed screens (two meshes to the inch) were used, thereby facilitating the passage of fecal particles through the screen. We have noted that animals receiving a vitamin B-deficient diet tended to be much more coprophagous than those which are fed a diet adequate in vitamin B but deficient in vitamin G. These and other observations suggested the necessity for further investigations relative to the causes of the above irregularities.

Sufficient experimental evidence (1-22) has been published to demonstrate that the vitamin B complex can be synthesized by microorganisms and that coprophagy may affect growth response to a marked degree.

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The experiments described in the present paper were conducted to determine the extent to which rats can synthesize vitamin B or vitamin G when the experimental diet is deficient in one or both of these factors.

#### EXPERIMENTAL

The basal diet consisted (in parts per 100) of washed casein 18, salt mixture (McCollum's 185 (23)) 4, agar 2, sucrose 15, dextrin 56, cod liver oil 2, and filtered butter fat 3. All the ingredients, with the exception of the cod liver oil and butter fat, were pulverized, mixed thoroughly, heated for 10 hours at 100–105° and stored in glass containers. The cod liver oil and butter fat were kept in a refrigerator and added to the basal diet, in the proportions indicated, just prior to the time of feeding.

The vitamin B supplement was prepared by percolating 95 per cent ethyl alcohol through dried brewers' yeast until the alcohol came through devoid of color. The percolate was concentrated under diminished pressure until the residue assumed a semisolid consistency. The residue was dried in a vacuum over sulfuric acid, macerated with cold 95 per cent alcohol, and filtered. The filtrate was made up to such a volume that 0.1 ml. of the solution represented 1 gm. of the original yeast.

The vitamin G supplement was made from bakers' yeast by adding ethyl alcohol to the aqueous extract of the yeast until the alcoholic concentration reached 50 per cent by volume. The precipitate obtained at this point was discarded, and the concentration of alcohol was increased to 80 per cent. A second precipitate was obtained, which was removed by filtration, dried, autoclaved for 6 hours at 15 pounds pressure, again dried, and pulverized. To facilitate the feeding of this fraction, it was diluted with powdered dextrin so that 0.3 gm. of the mixture represented 1 gm. of the original yeast.

Piebald rats, 20 to 21 days of age and weighing from 39 to 45 gm., were placed in individual cages provided, during the depletion period, with galvanized bottoms or grids which contained three meshes to the inch. At the end of the depletion period these screens were replaced by grids containing but two meshes to the inch. The cage pans were equipped with absorbent paper which absorbed the urine and facilitated the cleaning of the pans.

The animals were weighed at intervals of 1 week, at which times records were made concerning food consumption and physical condition. The time required for cessation of growth ranged from 10 to 24 days. At this point the animals were assigned to the various experimental groups, care being taken to observe the usual precautions regarding sex and litter origin.

TABLE I

*Effect of Vitamin B and Vitamin G Supplements on Rats of Series 1 with Small Meshed Screens Used during Depletion Period*

Group No.	No. of rats per group	Vitamin B concentrate fed daily	Vitamin G concentrate fed daily	Feces fed daily from group No.	Average initial weight	Weight at end of depletion period	Average gain in weight	Average weekly food intake	Rats surviving at end of experiment
		ml.	gm.		gm.	gm.	gm.	gm.	
1	4	0.1	0.3		40	47	93	39	4
2	4		0.3	8	42	58	78	37	4
3	4	0.1		8	41	48	73	38	4
4	4		0.3	9	41	54	69	39	4
5	4	0.1		9	40	56	66	37	4
6	4			9	40	47	45	28	4
7	4			8	42	51	38	26	4
8	12	0.1			40	46	19	28	12
9	16	*	0.3		41	49	15	30	14
10	4				41	50	0	12	0†

\* Each rat of this group received, weekly, 0.1 ml. of the vitamin B concentrate to prevent death from beriberi.

† All rats of this group died between the 27th and the 49th day after being placed on the basal diet.

The first series of experiments consisted of ten groups of animals which were fed the various supplements to the basal diet according to the outline given in Table I.

The animals in Groups 8 and 9 were placed on experiment from 12 to 20 days earlier than those of Groups 2 to 7 in order that their feces might be available for study with the last mentioned groups. The feces from animals in Groups 8 and 9 were collected from one to three times a week and stored under ether until the end of each experimental week. At this time the feces were pulverized and washed four times with ether. After the retained ether had been allowed to evaporate the finely pulverized material was stored in

glass containers. The daily allotment per rat was equivalent to the average daily amount of feces voided per day during the corresponding experimental week by each animal in Groups 8 and 9. The average daily amount of excreta voided per rat varied from 0.17 to 0.28 gm. of dry extracted feces. To facilitate the weighing of the daily allotment, sufficient powdered dextrin was

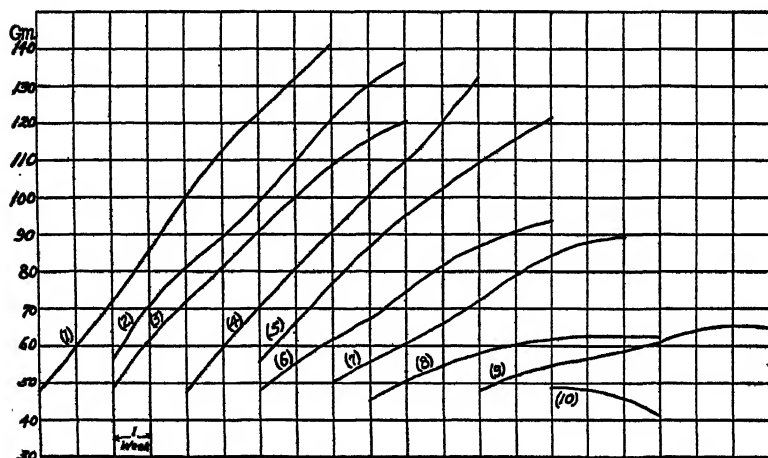


CHART 1. Growth response made by the several groups of rats constituting Series 1, with small meshed screens used during the depletion period. Group 1, basal diet + vitamins B and G; Group 2, basal diet + vitamin G + feces from Group 8; Group 3, basal diet + vitamin B + feces from Group 8; Group 4, basal diet + vitamin G + feces from Group 9; Group 5, basal diet + vitamin B + feces from Group 9; Group 6, basal diet + feces from Group 9; Group 7, basal diet + feces from Group 8; Group 8, basal diet + vitamin B; Group 9, basal diet + vitamin G; Group 10, basal diet alone.

added so that 0.3 gm. of the mixture represented the average daily elimination per rat during the preceding week.

The daily supplements were fed in special receptacles separate from the basal diet for a period of 8 weeks following the depletion period. The results obtained are given in Table I and Chart 1. Complete food intake records are omitted here, since food intake and growth response paralleled each other in a markedly uniform manner.

Examination of Chart 1 shows that the average body weights of the various groups at the beginning of the curative period were quite variable. This was due to the fact that the animals showed considerable variations in the time required for depletion. It was thought that these variations might be explained on the assumption that some coprophagy had occurred during the depletion period due to the small size of openings in the screens or grids. It was for this reason that the larger meshed screens were introduced

TABLE II

*Effect of Vitamin B and Vitamin G Supplements on Rats of Series 2 with Large Meshed Screens Used during Entire Experiment.*

Group No.	No. of rats per group	Vitamin B concentrate fed daily	Vitamin G concentrate fed daily	Feces fed daily from group No.	Average initial weight	Weight at end of depletion period	Average gain in weight	Average weekly food intake	Rats surviving at end of experiment
		ml.	gm.		gm.	gm.	gm.	gm.	
1	4	0.1	0.3		41	49	86	33	4
2	4		0.3	8	40	52	69	31	4
3	4	0.1		8	39	53	68	36	4
4	4		0.3	9	42	54	66	38	4
5	4	0.1		9	40	52	65	38	4
6	4			9	41	53	60	31	4
7	4			8	39	51	58	33	4
8	12	0.1			42	54	13	23	12
9	12	*	0.3		42	52	12	22	12
10	4				42	49	0	14	0†

\* All rats of this group received just sufficient vitamin B concentrate to prevent death from beriberi during the experimental period.

† All rats of this group died between the 34th and the 43rd day after being placed on the basal diet.

at the beginning of the curative period in Series 1. In order to obviate the discrepancies mentioned above, a second series of experiments was conducted in which the large meshed screens (two meshes to the inch) were used throughout the entire experimental period.

The details of management, feeding, etc., for Series 2 were identical with those described for Series 1. At the end of the depletion period (which was much more uniform than that of Series 1) the animals were divided into ten groups, after the usual

precautions regarding litter and sex distribution were observed. The diet and supplements of the various groups were identical with those of Series 1, as is shown in Table II, with the exception of Group 9. Instead of administering a weekly supplement of vitamin B to each animal as in Series 1, the supplement was not added to the diet until the animals had shown definite symptoms

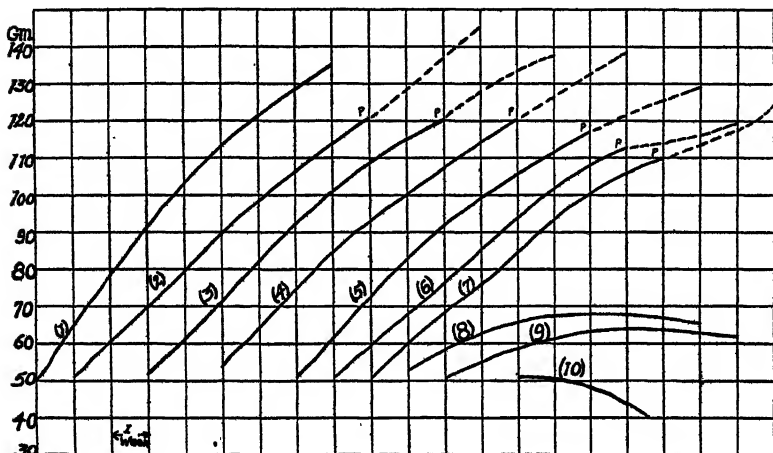


CHART 2. Growth response made by the several groups of rats constituting Series 2, with large meshed screens used during the entire experiment. Group 1, basal diet + vitamins B and G; Group 2, basal diet + vitamin G + feces from Group 8; Group 3, basal diet + vitamin B + feces from Group 8; Group 4, basal diet + vitamin G + feces from Group 9; Group 5, basal diet + vitamin B + feces from Group 9; Group 6, basal diet + feces from Group 9; Group 7, basal diet + feces from Group 8; Group 8, basal diet + vitamin B; Group 9, basal diet + vitamin G; Group 10, basal diet alone. P = changed to feces of rats receiving adequate amounts of vitamins B and G daily.

of vitamin B deficiency and the amount fed, from this point, was the minimum amount required to relieve paralytic symptoms and maintain life without producing a pronounced effect on growth.

The experiments in Series 2 differed in one other respect; *viz.*, after the usual 8 week curative period, Groups 2 to 7 received daily supplements of 0.3 gm. of extracted feces obtained from rapidly growing rats which had received daily allotments of dried bakers'

yeast. The data obtained by this change in feeding procedure are indicated in Chart 2 by the broken line extensions of the growth curves.

At the time these data were being assembled for publication, Roscoe's paper (24) appeared, emphasizing the significance and importance of coprophagy and refection in experimental rats. Our findings with few exceptions are in general agreement with those of Roscoe, although the methods of attack are somewhat different.

#### DISCUSSION

The marked similarity in results obtained in Series 1 and 2 (Charts 1 and 2) indicates that the variability in depletion time and in body weight at the beginning of the curative period in Series 1 had no appreciable effect in the subsequent deportment of the experimental animals. It would appear, therefore, that it is possible to discuss the results in terms of corresponding groups in both series of experiments.

The animals receiving the supplemented basal ration (Groups 10) responded in much the same manner, with the exception that those in Series 1 died between the 27th and 49th days, while those of Series 2 all died within a 9 day period, *viz.*, between the 34th and 43rd days of the experiment. Some of the animals manifested characteristic paralytic symptoms just prior to death, while others died without showing these symptoms. After death, however, the latter were found clutched, by mouth or by paws, to the side or bottom of the cage. The groups which received the vitamin G supplement (Groups 9) averaged 15 and 12 gm., respectively, in the 8 week period, and paralysis was observed quite frequently, especially in Series 2.

When vitamin B was the sole supplement to the basal ration (Groups 8) better initial growth was obtained, although there was a tendency for weight to decline toward the end of the 8 week experimental period. Many of these animals developed scaly feet and a mild form of dermatitis on the head and neck.

The groups which received the basal ration supplemented by feces from vitamin G-deficient animals (Groups 7) made an average gain of 5 and 7 gm. per week, respectively, and all animals remained in apparent good health until the experiment was ter-



minated. Our observations differed from those of Roscoe (24) in that we failed to note any abnormal size or volume of the feces other than that which could be explained on the basis of increased food intake. We noted that these animals tended to develop rough or shaggy fur.

The groups which received the basal ration supplemented by feces from vitamin B-deficient animals (Groups 6) grew at a rate quite comparable with that shown by Groups 7. Comparison of Curves 10 (Charts 1 and 2) shows quite clearly that diets deficient in vitamins B and G can be supplemented to advantage with feces. The addition of feces from normal rats receiving 0.6 gm. of yeast per day did not prove beneficial so far as stimulation of new or more vigorous growth was concerned.

Groups 5 which received the basal ration supplemented with the vitamin B concentrate and feces from Groups 9 showed an average weekly gain of about 8 gm. The response of Groups 4 was quite comparable with that of Groups 5.

The results obtained in Groups 3 show how feces from vitamin G-deficient rats can supplement a vitamin G-deficient diet. Groups 2 grew well on a diet deficient in vitamin B when the diet was supplemented by feces from rats which had received adequate amounts of this vitamin.

Our data are in general agreement with those of Roscoe (24) regarding the stimulatory effect of feces. The growth responses in Groups 2 to 7 show quite conclusively that vitamins B and G were supplied from feces in sufficient amount to produce satisfactory growth. We are unable to explain the method or mechanism by which vitamins B and G are synthesized. It is possible that feces contain a substance or substances which encourage the growth of bacteria in the intestine. This has been suggested by Roscoe (24). Kennedy and Palmer (18) believe that the stimulatory effect induced by coprophagy is due to the presence of an unknown vitamin or substance which stimulates growth of the rat. Roscoe states that sterilization of feces did not destroy the growth-promoting properties. If this is true it would appear that growth stimulation cannot be due to reinfection. Since all existing data indicate that body storage of the vitamin B complex is quite limited, we are forced to conclude that the beneficial effect of coprophagy is not due solely to the excretion of the original body

stores of vitamins B and G as was indicated in a previous publication from this laboratory (8).

The responses obtained in Groups 3 and 4 indicate quite clearly that both vitamins are synthesized in the body. This is borne out by the fact that good growth was obtained when feces from vitamin B- and G-deficient animals were used to supplement vitamin B- and G-deficient diets. Our data seem to indicate that the relative quantities of these vitamins eliminated in feces are approximately equal and that the amount eliminated is independent of the limiting factor in the diet. A number of observations have been made which show that animals receiving the basal diet without supplement, eliminate feces which are potent in both factors.

Four male rats (Rats 6479, 6480, 6481, and 6482), all from the same litter, were placed in the usual type of cages and were fed the basal diet. The records of their dietary régime and responses are recorded here, since they are not included in Tables I and II and Charts 1 and 2.

Rat 6479 weighed 45 gm. when placed on the basal diet and reached its maximum weight of 64 gm. by the 14th day. From this point the weight of the animal gradually decreased, with symptoms of beriberi appearing on the 35th day and death occurring on the 43rd day.

Rat 6480 weighed 42 gm. when placed on the basal diet and gained 10 gm. during the first 14 days. On the 28th day it manifested symptoms of beriberi and was given daily, during the next 14 days, its own feces eliminated during the previous 24 hours. Its weight increased from 48 to 67 gm. during this time. The feces feeding was discontinued during the following 14 days and the weight of the animal decreased to 50 gm., without any apparent symptoms of beriberi being observed. The feces were again fed for 14 days, as previously indicated, and the weight increased to 85 gm.

Rat 6481 weighed 43 gm. when placed on the basal diet and attained a weight of 52 gm. by the 14th day, at which time the weight began to decline. On the 29th day the animal weighed 46 gm. and showed marked symptoms of beriberi. It was given its own feces for 14 days. During this time the paralytic symptoms disappeared and the weight increased to 77 gm. The feces feeding

was discontinued from this point and body weight decreased gradually until death resulted on the 77th day. At this time the animal weighed 40 gm. and had manifested slight symptoms of beriberi.

Rat 6482 weighed 40 gm. when placed on the basal diet and made a 9 gm. increase during the first 14 days on this diet. Paralytic symptoms were noted on the 28th day, at which time the animal was fed its own feces for the next 14 days. During the 14 days in which the feces supplement was fed, the paralytic symptoms disappeared and body weight increased from 42 to 57 gm. The feces supplement was then omitted from the diet and the weight of the animal decreased gradually until the 65th day, when death occurred. At the time of death the animal weighed 36 gm.

Thus, as a result of receiving its own feces as a supplement to a vitamin B complex-deficient diet, each of three animals showed a definite increase in weight and a marked improvement in physical condition, while their litter mate, which did not receive the supplement, grew gradually weaker and died at an earlier date.

While our studies do not answer the question as to the mechanism of growth stimulation in coprophagous rats, they seem to show that studies involving the assay of foods for vitamins B and G can be vitiated by the introduction of adequate amounts of these vitamins if coprophagy is permitted. Coprophagy can be reduced to a minimum if screens with large meshes are used. Accurate experiments involving assays for vitamins B and G may necessitate a series of screens of different sized meshes to be changed from time to time as the animals increase in size. The question of coprophagy still remains a serious problem and merits continued study. We hope to continue the study with the view of investigating the bacteriological phases of the problem.

#### SUMMARY

1. A quantitative study is described in which rats were fed diets deficient in vitamins B and G, respectively. Feces from these rats were fed to other rats as supplements to diets which were also deficient in these vitamins.

2. Rats which received diets deficient in vitamins B or G eliminated feces which were potent sources of these vitamins.

3. The potency of the feces eliminated by vitamin B- and vita-

min G-deficient rats showed no tendency to become less potent as the experiments progressed.

4. The amounts of vitamins B and G found in feces seem to be about equal and do not seem to depend on the diet of the animal.

5. No satisfactory explanation can be furnished at the present time regarding the mechanism whereby these vitamins are synthesized.

6. The danger of coprophagy, as it affects the accuracy of assays of vitamins B and G, is discussed.

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## DETERMINATION OF IRON IN COW'S MILK AND HUMAN MILK

BY FREDERICK REIS AND H. H. CHAKMAKJIAN

(From the Department of Biological Chemistry, Tufts College Medical School, Boston)

(Received for publication, July 6, 1932)

In a previous paper<sup>1</sup> a method was described by us for the determination of iron in blood as dispersed Prussian blue. We have applied the method to the determination of very small amounts of iron in various substances, organic and inorganic. The application of the method to the analysis of milk is reported in the present paper. The solutions needed are the same as given in the previous article. The preparation of standard ferric sulfate solution, we have found by extensive trials, will be more reliable if ferrous ammonium sulfate (Mohr's salt), which is stable under all atmospheric conditions, is employed. It must, however, be oxidized to the ferric state by 0.2 N potassium permanganate solution prior to use as a standard solution.

7.0226 gm. of c.p. ferrous ammonium sulfate are dissolved in about 50 cc. of water and 10 cc. of concentrated sulfuric acid. The hot, nearly boiling solution will take about 18 cc. of 0.2 N permanganate solution for oxidation. The oxidized solution, after cooling, is transferred to a liter flask, diluted with the washings, and made up to the volume. This is a stock solution containing 1 mg. of iron per cc., 10-fold dilution of which will make the standard solution. We have checked repeatedly this standard solution against one prepared from analytical iron by colorimetric and titration methods.

A change in the type of digestion tube has also been made. We are now using a Pyrex micro-Kjeldahl flask graduated on the stem to 10, 15, and 25 cc. Because of its long stem this type of

<sup>1</sup> Reis, F., and Chakmakjian, H. H., *J. Biol. Chem.*, **92**, 59 (1931).

tube facilitates the digestion and dilution of the digested product to proper volume, which dilution depends on the approximate iron content of the sample. It also eliminates the necessity of preparing several standard solutions, as well as undue dilution of the Prussian blue solution.

The iron content of milk is of physiological interest. It is the only source of iron for the child for about 10 months.

After the analysis of a few samples of milk bought from a store it occurred to us that part of the iron, small as it was, might have come from the tin container used in collection of milk at the farm. We therefore obtained a sample of milk from four cows milked directly into a bottle. The analysis of the latter seemed to justify our suspicion that a small part of iron in milk owes its source to rusty cans.

### *Procedure*

Transfer 5 cc. of milk to a digestion tube, add 1 cc. of concentrated sulfuric acid, and evaporate carefully. The liquid froths freely, but by heating the uppermost part of the foam the loss of the liquid is prevented. When the thick fumes of the acid appear, add 1 cc. of fresh acid, heat for a minute, and add slowly 2 cc. of saturated solution of potassium chlorate; heat 3 minutes and again add, drop by drop, 1 cc. of potassium chlorate solution; heat vigorously for 2 minutes, continue the additions of chlorate, 1 cc. at a time, and heat each time for 2 minutes until the color of the liquid is brown. A small funnel in the stem of the digestion tube serves as a condenser for the fumes of the acid, and a larger funnel inverted over the small one removes uncondensed fumes by suction.

Add 1 small drop of 0.2 N permanganate solution, followed by 1 cc. of concentrated sulfuric acid and 1 cc. of chlorate. Add more chlorate until the color of permanganate reappears for a few seconds. At this point most of the organic matter is destroyed, except a very small amount of some resistant compound, possibly fatty acid, which will cause turbidity in dispersed Prussian blue.

To insure complete oxidation add, slowly, 2 cc. of water, 4 or 5 drops of 10 per cent glucose or sucrose solution, and 1 cc. of chlorate solution; heat until thick fumes of acid are produced and continue boiling gently for 5 minutes, or until the residual thick liquid

Sample No. and source of milk	Amount taken	Method of analysis	Iron found, mg. per 100 cc. sample
	cc.		
1 (store)	5	Colorimetric	1.00
	3	"	0.97
2 "	7	"	0.28
	10	Titration with titanium chloride	0.26
3 "	5	Colorimetric	0.38
	5	"	0.38
	5	"	0.40
	5	"	0.39
	5	"	0.42
4 "	5	"	0.45
	5	"	0.44
5 "	5	"	0.21
	15	Titration	0.25
6 (local farm)	5	Colorimetric	0.33
	5	"	0.36
	5	"	0.35
	gm.		
Same after souring,	5	"	0.33
jelly-like mass	5	"	0.32
	cc.		
7 (local farm)	5	"	0.28
	5	"	0.27
	15	Titration	0.26
8, milk directly from 4 cows	5	Colorimetric	0.15
	5	"	0.14
Same after souring, jelly-like mass	10	Titration	0.16
	10	"	0.17
Whey of same	15	"	0.110
	15	"	0.110
	15	Colorimetric	0.109
	15	"	0.108
9, human	5	"	0.34
	5	"	0.33
10, "	5	"	0.39
	5	"	0.39
11, "	10	"	0.32
	10	"	0.31
12, "	5	"	0.37
	5	"	0.36
13, "	5	"	0.45
	5	"	0.45
	5	Titration	0.45
14, "	10	"	0.35
15, "	5	Colorimetric	0.29
	5	"	0.29



is clear. It may take about 15 cc. of chlorate solution. Cool and dilute to about 5 cc. If the solution is clear on cooling, add 0.2 cc. of gum ghatti-potassium ferrocyanide solution and dilute to the 10 cc. mark.

Prepare the standard solution in a 50 cc. flask; to 1 cc. of standard iron solution add 2 cc. of concentrated sulfuric acid, 2 cc. of a 10 per cent solution of ammonium sulfate to compensate the ammonium salt obtained from the milk, 10 to 15 cc. of 6 per cent potassium sulfate; cool; add 1 cc. of gum ghatti-potassium ferrocyanide solution, mix well, and dilute to the 50 cc. mark. Shake both solutions vigorously for a few minutes, allow them to stand for 15 minutes, compare the colors, taking the usual precautions, reading standard against standard, etc.

For example, the digestion product from 10 cc. of milk was converted into 15 cc. of Prussian blue solution; the standard was diluted to 50 cc.

With the standard set at 20 (or a point near 20 to produce equal color with the standard on the right set at 20), the reading of the unknown was 17.5, the ratio of dilution being 0.3.

$$\frac{20 \times 0.1 \times 0.3}{17.5} = 0.0343 \text{ mg. of Fe in 10 cc. of milk, in 100 cc.}$$

0.34 mg., actually 0.32 mg. after subtracting the amount of iron found in the reagent used.

In our percentages we omitted the third decimal figures, which come within the limits of the experimental error.

The results of our experiments are given in Table I.

## THE ACID-BASE EQUILIBRIUM IN ABNORMAL PREGNANCY

By DAVID M. KYDD,\* HARRY C. OARD,† AND JOHN P. PETERS

(From the Department of Internal Medicine of Yale University and the Medical Service of the New Haven Hospital, New Haven)

(Received for publication, September 9, 1931)

In previous papers (9, 14) we have discussed the changes that occur in the acid-base equilibrium of serum of normal women during pregnancy. In brief, these changes are reduction of the serum carbon dioxide content and serum proteins, accompanied by a comparable reduction of total base. These findings have been confirmed by Stander, Eastman, Harrison, and Cadden (21). The reduction of serum proteins appeared to be at the expense of the albumin fraction (5, 9, 17). Stander *et al.* (21) reported a normal hydrogen ion concentration and this has been confirmed by us (9). Thus the reduction of the carbon dioxide content of the serum, which has long been known to occur during the course of normal pregnancy, is associated with a diminished total base and not an accumulation of other "acids."

The explanation of these abnormal findings remains obscure and it was felt that a study of abnormal pregnancy might contribute not only to the solution of the pathogenesis of the toxemias themselves but also to the explanation of the acid-base disturbances in normal pregnancy.

During the course of pregnancy there may occur one of a group of disorders, loosely termed the toxemias of pregnancy. Because of their obscure etiology as well as clinical diversity, the classification of the toxemias has been difficult and confused. The numer-

\* Holder of Sterling Research Fellowship at Yale University, 1929-1930, whose work was further assisted by a grant from the Sax Research Fund.

† Part of this work was done by Dr. Harry C. Oard in fulfillment of the thesis requirement for the Degree of Doctor of Medicine at Yale University School of Medicine.

ous investigations of these disorders have been confined mainly to determinations of blood carbon dioxide and nitrogenous constituents. These investigations have been reviewed by Stander (19). Because they have consisted of more or less isolated observations discussed without proper relation to the changes found in normal pregnancy they have not aided in the solution of the general problem. Stander *et al.* (20, 21) and Muntwyler, Limbach, Bill, and Myers (13) have described complete acid-base studies in abnormal pregnancy. These will be discussed below. In this paper are reported studies of the complete acid-base equilibrium of the serum of patients with abnormal pregnancy during the acute phase and, in so far as was possible, after recovery.

### *Methods*

The methods for withdrawal of blood and the analytical procedures and calculations utilized in these investigations have been described in previous papers from this laboratory (9, 14). The pH was determined gasometrically, with the value for  $pK'_1$  of 6.12. The vomitus was analyzed by techniques similar to those used for serum. In the less acute cases the blood was withdrawn in the morning before the patient's breakfast. In cases with convulsions or severe vomiting the first blood sample was withdrawn as soon as possible after the patient entered the hospital, before treatment was instituted.

### *Results*

Results of the analyses are presented diagrammatically in Charts I to III. The acid anions actually determined by analysis were bicarbonate, chloride, phosphate, and protein. The sum of these subtracted from the total base, the undetermined acids, is presumably composed of sulfates and organic acids. In the absence of evidence that sulfates accumulate in excess in the serum in pregnancy, it may be assumed that changes of the undetermined acid fraction are referable entirely to organic acids. The total height of each column on Charts I to III represents the amount of total base found. This determination, for brevity, is not separately indicated. For comparison there have been included with each chart columns representing the normal non-pregnant and normal pregnant states. The chief clinical symptoms of each

patient are shown on Charts I to III. The clinical classification of Stander (19) has been employed.

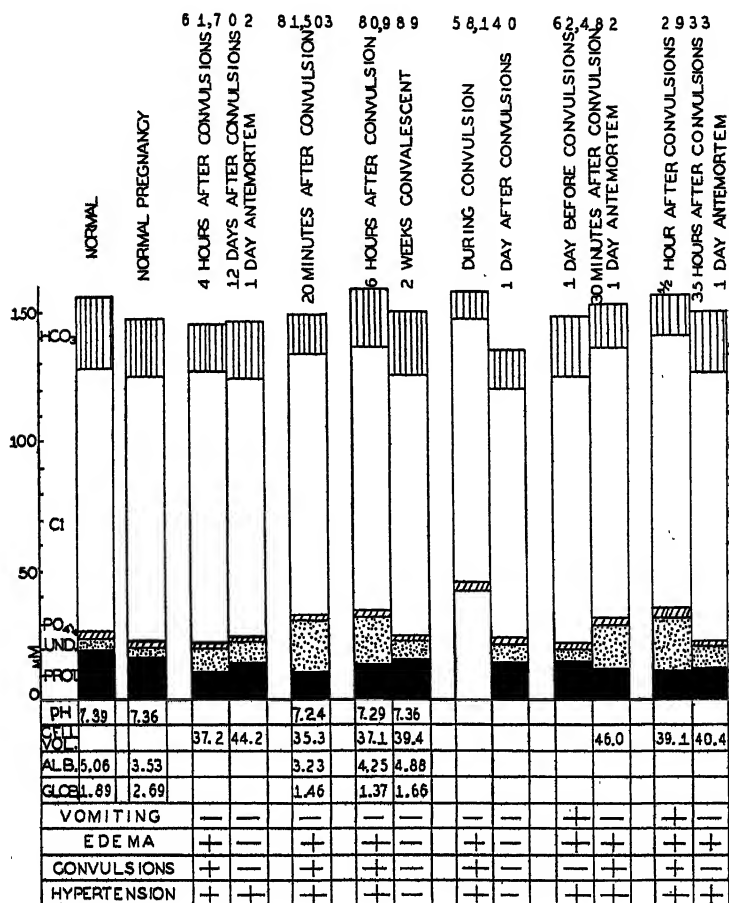


CHART I. The acid-base balance in eclampsia. Alb. = albumin expressed in gm. per cent; glob. = globulin expressed in gm. per cent; und. = undetermined acids. When the clinical manifestations noted on the left occur, there is a plus sign (+); when absent, a minus sign (-). The columns representing the acid-base balance in normal individuals and normal pregnant individuals were constructed from figures previously published (9, 14, 15). The figures given above the columns represent the respective case numbers.

*Eclampsia*

Chart I represents the findings in six cases of eclampsia, three of which proved fatal. In brief, the deviations from the normal pregnant acid-base balance in the acute stage of the condition are: a considerable lowering of the carbon dioxide content, accompanied by an elevation of the total base, chlorides, and undetermined acids of the serum. The proteins appear to be reduced at the time of the convulsive seizure. There are, unfortunately, but two observations of pH, which was definitely lowered on both occasions. Stander (21) has reported five cases of eclampsia with results quite similar to ours except that the pH values are much lower, due probably to the fact that he withdrew the blood practically during convulsions. Even in his cases the changes are no greater than those found by Barr (2) during severe muscular activity. It is to be noted that in Case 80,989 there is practically no reduction of  $\text{CO}_2$ . This case, having received large doses of morphine, had been without convulsions for several hours. Henderson and Haggard (8) and also Cobet (4) found, after large doses of morphine, high  $\text{CO}_2$  and low pH, which they ascribed to reduction of the respiratory rate. It is our belief that the disturbances described are the effect rather than the cause of the convulsions. In one case, Case 62,482, blood was obtained the day before any convulsions had occurred. Although the patient exhibited all the premonitory symptoms of eclampsia, the serum acid-base equilibrium was that of normal pregnancy.

In their last report Stander *et al.* (20) suggest that the acidosis associated with eclampsia may be a cause of the fatalities. In two of our fatal cases (Cases 62,482 and 2933), samples of blood were obtained shortly before death, but after the convulsions had ceased. In these two instances the acid-base equilibrium no longer had acidotic characteristics but had become more like that of normal pregnancy. The third fatal case (Case 61,702) survived 10 days after delivery and the last convulsions. In this case also the deviations of the acid-base balance found during the convulsive period disappeared after recovery from this phase. These observations strongly indicate that the acidosis is referable to the symptoms, convulsions, rather than to the underlying pregnancy toxemia.

The reduction of serum proteins during the convulsive stages of

eclampsia is not so simply explained. Analysis of the protein fractions in Case 80,989 indicate that both albumin and globulin are affected. This would suggest that the reductions were due

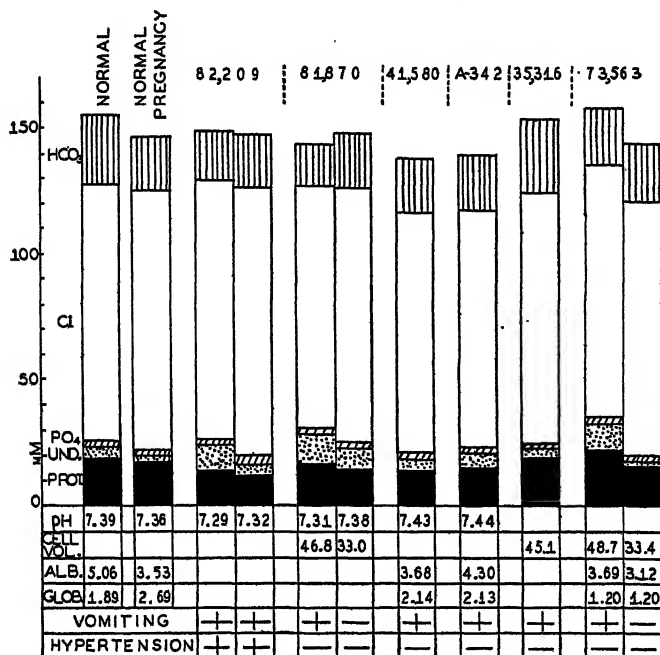


CHART II. The acid-base balance in vomiting of pregnancy. Alb. = albumin expressed in gm. per cent; glob. = globulin expressed in gm. per cent; und. = undetermined acids. When the clinical manifestations noted on the left occur, there is a plus sign (+); when absent, a minus (-). The columns representing the acid-base balance in normal individuals and normal pregnant individuals were constructed from figures previously published (9, 14, 15). In each case the first column represented the electrolytes found before treatment was instituted; the second, after recovery, except in Case 82,209 in which each column represents the electrolytes found before treatment on two separate admissions. The figures given above the columns represent the respective case numbers.

to temporary hemodilution. Blood cell volumes, determined in Cases 61,702, 80,989, and 2933 indicate hemodilution, despite the increase in total base and other ions noted above. The phenomenon deserves more careful analysis, perhaps with direct

determinations of blood volume. Comparison with other convulsive states would also be desirable. That the protein reductions are connected with the convulsive phase of the condition seems to follow from the fact that proteins were lower during, than either before or after, this phase (compare Case 62,482 with Cases 61,702, 80,989, and 2933).

### *Vomiting of Pregnancy*

The findings in six cases of vomiting of pregnancy are represented in Chart II. In these cases of severe and protracted vomiting, blood cell volume and serum protein are elevated, evidencing dehydration. The carbon dioxide content is depressed and the undetermined acids are increased. This condition is usually

TABLE I  
*Total Base and Chloride Analysis of Vomitus Obtained during Pregnancy*

Case No.	Total chloride	Total base
	<i>mm</i>	<i>mm</i>
41,580	49.8	73.5
81,870	52.0	52.0
A-342	72.2	92.0
97,261	79.0	78.2
97,261	15.2	15.3

In none of the specimens was there any free hydrochloric acid.

accompanied by the presence of acetonuria and other evidences of starvation. The serum chloride is either normal or low. If low, it appears to be accompanied by a low total base. The pH is in the normal range in all cases. Because these results differ very markedly from those found in cases of protracted vomiting caused, for example, by pyloric obstruction, the vomitus in these cases was examined (Table I). In all instances the amount of chloride found in the vomitus was equaled or exceeded by the amount of total base. In none of the specimens was there any free hydrochloric acid. Arzt (1), in studying the gastric secretion, found free hydrochloric acid absent in twenty-nine out of 50 cases of early pregnancy and diminished in all the remaining cases. This finding of an equivalent amount or an excess of total base in relation to the amount of chloride lost in the vomitus accounts

for the electrolytic picture observed. Following treatment and relief of vomiting, the serum assumes the characteristics found in normal pregnancy.

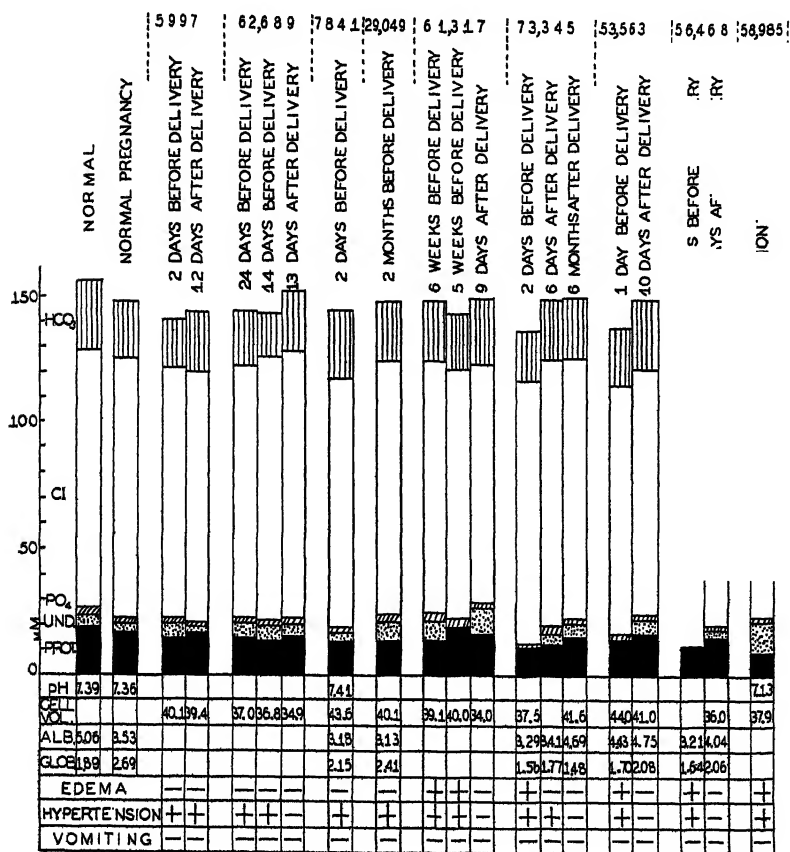


CHART III. The acid-base balance in "low kidney reserve." Alb. = albumin expressed in gm. per cent; glob. = globulin expressed in gm. per cent; und. = undetermined acids. When the clinical manifestations noted on the left occur, there is a plus sign (+); when absent, a minus (-). The columns representing the acid-base balance in normal individuals and normal pregnant individuals were constructed from figures previously published (9, 14, 15). The last column represents a case of nephritis complicated by pregnancy. The figures given above the columns represent the respective case numbers.



*Low Kidney Reserve*

In Chart III are presented the data from cases with "low kidney reserve." In order to conform to the classification given by Stander (19) all but one of the cases not already considered have been placed in this group. This we found somewhat unsatisfactory as there are actually two types of cases, namely those with edema and those without. Because of the overlapping, it is difficult completely to separate the two. The cases without edema show no deviation of the electrolyte picture from that of normal pregnancy and the pH is also normal. In edematous patients the serum proteins are reduced below the level found in normal pregnancy, but the electrolyte picture is otherwise undisturbed. These findings agree with those of Stander (20, 21).

The case represented by the last column on Chart III is a case of chronic nephritis complicated by pregnancy. Here the deviations from the normal are well marked but simulate very closely the changes described as occurring in nephritis of the same degree in the absence of pregnancy.

## DISCUSSION

The observations reported give no indication that any type of toxemia *per se* is associated with a characteristic electrolyte disturbance. Deviation of the acid-base and electrolyte patterns from those of normal pregnancy appear to be related to symptoms which the patients exhibit rather than to the underlying disease. With relief of these symptoms the deviations become negligible.

During eclamptic convulsions there is an acidosis similar to that of severe exercise.

In vomiting of pregnancy the electrolyte picture differs from that usually encountered in vomiting states only because the vomitus is deficient in hydrochloric acid. Base is lost in amounts equaling or exceeding those of chloride. This, together with the accession of ketone acids resulting from starvation, causes a carbon dioxide deficit instead of the carbon dioxide excess found in patients who vomit an excess of hydrochloric acid.

The excessive reduction of proteins found in patients with edema may be attributable to the drain of proteinuria added to the unknown factors which are responsible for the slight protein deficiency of normal pregnancy. The protein deficits are not,

however, comparable in degree to those which are necessary for the production of edema in nephritis (12, 16), malnutrition (3, 11, 22), or after phasmapheresis (10); and would seem, therefore, to act merely as a contributory factor in the pathogenesis of edema during pregnancy.

The results of these studies are in general agreement with those of comparable investigations made by Stander (20, 21), however conclusions concerning their cause and significance may differ. Muntwyler, Limbach, Bill, and Myers (13) have recently reported similar analyses from twenty-six cases of abnormal pregnancy. Their results are in accord with ours, with one exception: They found the average serum pH in their cases distinctly elevated. They conclude that the acid-base disturbances are due to hyperventilation. This, they believe, lowers the  $\text{CO}_2$ , the base falling secondarily as a compensatory reaction. Not content with confining these conclusions to pathological cases, they extend them to cover the changes of normal pregnancy, without presenting any data from uncomplicated cases.

These conclusions are open to serious objections on several grounds. In the first place the discovery of high pH is at variance with the results of observations by the gasometric method reported in this paper and a preceding paper from this laboratory (9), dealing with pathological and normal pregnant subjects respectively, and with the electrometric determinations on normal and pathological pregnant subjects published by Stander *et al.* (20, 21), Rolly (18), and others. In the second place Muntwyler's paper deals with heterogeneous material of great variability. His pH values are not consistent, but there are no clinical records to facilitate analysis of the results. Averages from such data can have little significance.

Finally, Muntwyler finds by a similar treatment of averages, that the elevation of pH persists for some days after the termination of pregnancy, although base and  $\text{CO}_2$  have returned to the normal level. This would in any case invalidate his theory concerning the cause of the  $\text{CO}_2$  and base deficits, because the high pH is evidence that the hyperventilation persists after the  $\text{CO}_2$  deficit, to which it is supposed to have given rise, has disappeared. It may not be amiss to point out in this connection, also, that no one has yet demonstrated that the  $\text{CO}_2$  deficit of hyperventilation

is compensated by base reduction. Some experiments on the subject by Peters, Bulger, Eisenman, and Lee (15) and others (6, 7) indicate that compensation for such  $\text{CO}_2$  deficit is established by increase of chloride without change of base.

#### SUMMARY

1. In pregnancy toxemias disturbances of the electrolyte and acid-base equilibria in the serum appear to be referable to symptoms of the conditions rather than to the fundamental character of the toxemias.

2. During the convulsions of eclampsia the changes in serum electrolytes are similar to those found in severe exercise: reduction of  $\text{CO}_2$  and pH, with increase in base, Cl, and organic acids. Protein is also slightly diminished.

3. In severe vomiting  $\text{CO}_2$  falls. The explanation for this is found in the low acidity of the vomitus.

4. Serum proteins are lower in edematous cases than in non-edematous cases or normal pregnancy.

For the opportunity to study these cases and cooperation in securing the data we are indebted to the Department of Obstetrics and Gynecology and especially to Professor Arthur H. Morse.

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## THE ACID-BASE BALANCE DISTURBANCE OF PREGNANCY

BY VICTOR C. MYERS, EDWARD MUNTWYLER, AND ARTHUR H. BILL

*(From the Departments of Biochemistry and Obstetrics, School of Medicine, Western Reserve University and the Maternity Hospital, Cleveland)*

(Received for publication, November 6, 1931)

In the preceding paper by Kydd, Oard, and Peters (1), which we were given an opportunity to review, these authors take exception to several statements in a paper by Muntwyler, Limbach, Bill, and Myers (2). They state, referring to this paper:

"Their results are in accord with ours, with one exception: They found the average serum pH in their cases distinctly elevated. They conclude that the acid-base disturbances are due to hyperventilation. This, they believe, lowers the  $\text{CO}_2$ , the base falling secondarily as a compensatory reaction."

In our paper (2) it was stated:

"With this decrease in base there was also a tendency to a definite increase in pH. The maximum antepartum pH finding was 7.53 with an average of 7.47. This would indicate that hyperventilation was a factor in the bicarbonate decrease observed. This supposition is further supported by the decrease in chloride post partum associated with the increase in bicarbonate. As was mentioned previously, the assumption of hyperventilation as the main cause of the bicarbonate decrease is in agreement with the views of Austin and Cullen who based their opinion on the increased pH observed by Marrack and Boone, and with the views of Gaebler and Rosene, who found an increased plasma pH ante partum which returned to normal with the bicarbonate post partum."

Two statements in our summary bearing on this question might also be quoted:

"The pH was elevated to 7.48 or above in thirteen of the twenty-three cases studied ante partum, with an average pH of 7.47" and "From the data at hand it would seem most logical to conclude that the fall in the plasma

bicarbonate noted in the present series of cases of toxemia of pregnancy (and in normal pregnancy) is due to hyperventilation, and that the fall in total base is a further compensatory phenomenon."

In particular Kydd, Oard, and Peters (1) appear to object to our assumption that the fall in bicarbonate results from hyperventilation, *i.e.*, hyperventilation lowers the  $\text{CO}_2$ , the base falling secondarily as a compensatory phenomenon, but not always proportionately, and thus in many cases brings about a slight elevation of the pH.

Workers are generally agreed that in pregnancy there is a fall in bicarbonate. The question under dispute then is whether this fall in bicarbonate is brought about by a primary  $\text{CO}_2$  deficit or a primary alkali deficit; *i.e.*, whether there is a shift from Area 5 into Areas 2 and 3 or directly to Area 6 in the Van Slyke diagram (3). Without answering this question an adequate explanation of the so called "acidosis of pregnancy" cannot be given. We believe that there are a great many data supporting the  $\text{CO}_2$  deficit theory, but few or no data supporting the opposing view.

The evidence supporting the view that the fall in the blood bicarbonate during pregnancy results from hyperventilation will be presented briefly. Observations were reported a number of years ago by Magnus-Levy (4) and Zuntz (5), which show that there is a definite increase in the minute volume of air respired (20 to 30 per cent). Later it was shown by Leimdörfer, Novak, and Porges (6), Hasselbalch and Gammeltoft (7), Rowe (8), and others that there was a reduction in the  $\text{CO}_2$  tension of the alveolar air. In harmony with this observation a large number of investigators have uniformly found a reduction in the  $\text{CO}_2$ -combining power or  $\text{CO}_2$  content of the blood, following the introduction of a convenient method for this determination by Van Slyke. If this fall in  $\text{CO}_2$  is the result of hyperventilation, the excretion of an equivalent amount of base in the urine might produce complete compensation, or the excretion of base might lag behind the  $\text{CO}_2$ , thus shifting the hydrogen ion concentration slightly toward the alkaline side. In either case the reaction of the urine should become somewhat more alkaline than normal. Bokelmann and Rother (9) have recently presented convincing evidence that the pH of the urine is more alkaline during pregnancy than some time after delivery. It is a well known fact that with the loss of  $\text{Cl}^-$ , addi-

tional  $\text{HCO}_3^-$  may combine with base, and *vice versa*. It is probable that the Cl ion compensates to a certain extent for the loss of  $\text{CO}_2$  but not sufficiently to prevent a slight shift in the reaction of the blood. Although the change in the reaction (pH) of the blood is small, there is abundant and convincing evidence that a slight shift to the alkaline side does occur. It is sufficient to refer to the publications of Marrack and Boone (10), Gaebler and Rosene (11), and to the recent papers of Siedentopf and Eissner (12) and Wirz (13), all of whom are in agreement that there is a slight but definite tendency toward an elevation of the pH in normal pregnancy.

Since the slightly elevated pH is the point where our observations (2) and those of Kydd, Oard, and Peters (1) differ, it may be well to review the evidence on this point.

Kydd, Oard, and Peters (1) cite the observations of Stander, Eastman, Harrison, and Cadden (14) giving electrometric pH figures for three pregnant and three non-pregnant women, those of Kydd (15) presenting gasometric pH figures for ten pregnant and eight non-pregnant subjects, and three electrometric observations made by Rolly (16) in 1913 to prove that the pH remains unchanged during normal pregnancy.

Against these observations we wish to cite the four papers to which reference was made above, also some observations made by Michaelis in 1913 (17), all of which record an increase in the pH during normal pregnancy. Marrack and Boone (10) report the findings for sixteen pregnant women, while Gaebler and Rosene (11) present 88 observations on thirty-two normal pregnant young women. Both groups of workers employed the colorimetric method, Gaebler and Rosene carrying out their determination with the same technique employed in our analyses. Similar findings obtained with the electrometric method have recently been recorded. Siedentopf and Eissner (12) have reported observations on forty-eight pregnant women and six controls, while Wirz (13) has recorded figures for thirty pregnant and sixteen non-pregnant women. If one refers to the older literature, the observations of Michaelis (17) should not be overlooked. Electrometric pH figures for twenty-three pregnant women (chiefly during the early months of pregnancy) and for sixteen controls were given, the pregnancy bloods showing an average higher value of pH 0.054.



These investigators did not invariably find the pH elevated in all the observations made during pregnancy, but there was an appreciable elevation in a considerable number of cases, and this was sufficiently large to yield a higher average than normal. For example, the average in Wirz's group showed an increase of 0.03 pH over the controls. It is of interest that this same average difference can be found in the six cases reported by Stander *et al.* (14).

Attention should probably be called to the paper of Bock (18) who reports forty-two pH observations on pregnant women and six controls made electrometrically. The figures as given show a pH slightly more acid than normal during the last months of pregnancy. The six controls are not uniform and include one exceptionally high figure. However, if the figures are brought to the same temperature basis, they show a more acid reaction at term, but a slightly more alkaline reaction during the 2nd to the 5th month, at birth, and 8 days later.

Kydd *et al.* state (1): "Not content with confining these conclusions to pathological cases, they extend them to cover the changes of normal pregnancy, without presenting any data from uncomplicated cases." It will be noted that in the statement covering this point in our summary and quoted above, "(and in normal pregnancy)" is put in parentheses. This statement was intended to convey the impression that our observations were very similar to those found in normal pregnancy, and would apply equally well to them, although no data were included in the paper. There was also a further reason for making this statement. The work of Gaebler and Rosene (11) on the acid-base balance in normal pregnancy was carried out in the laboratory of the senior author, and our study (2) was considered in part as a continuation of this work. Kydd *et al.* (1) are apparently unaware of the paper of Gaebler and Rosene, although it appeared some months prior to the first publication of Oard and Peters (19) on the subject.

We wish to deny the statement that "Muntwyler's paper deals with heterogeneous material of great variability," also the statement that, "His pH values are not consistent." We believe that our pH findings varying from 7.39 to 7.53 with an average of 7.47 are very consistent, and are just what one might expect in normal pregnancy or in mild toxemias of pregnancy. Furthermore, they

are in harmony with the electrometric pH determinations presented in the comprehensive papers by Siedentopf and Eissner (12) and Wirz (13), who draw their conclusions in part from their average pH values, quite unaware of the objections of Kydd, Oard, and Peters (1) to such data.

The clinical records were purposely omitted from our paper for the simple reason that our findings were in essential harmony with those found for normal pregnancy by Gaebler and Rosene (11) and would, therefore, have consumed needless space in a biochemical journal.

The criticisms of Kydd, Oard, and Peters (1) are apparently directed primarily at our pH figures. Our forty-four pH observations were all made colorimetrically at 20° with the Cullen method as described by Myers and Muntwyler (20). The first ten of these observations were checked against the electrometric method with good agreement. The correction factor for these ten specimens averaged 0.223 compared with the Cullen factor of 0.22. The results obtained on these ten specimens (first six cases) with either the electrometric or colorimetric method would lead to the same interpretation. For reasons given elsewhere (21) we believe that the accuracy of the colorimetric method on these blood specimens obtained during pregnancy was equal to that on normal blood.

It would seem to us that Kydd *et al.* (1) have too few gasometric pH observations from which to draw conclusions. Owing to the large number of electrometric (and colorimetric) observations on record, the majority of which show an elevation in the pH, one is forced to the conclusion that either their gasometric pH determinations are not representative, or they do not agree with the electrometric determinations. Since the electrometric method has generally been taken as the method of reference, we do not see how Kydd *et al.* (1) can disregard these electrometric pH observations.

It might be noted that in Kydd's paper (15) on the hydrogen ion concentration and acid-base equilibrium in normal pregnancy Kydd presented but ten pH figures (gasometric) on blood specimens obtained during pregnancy. We gave the same number of electrometric pH figures in our Table I.

As we stated in our paper (2), a satisfactory explanation of the

slightly higher pH values found post partum in ten out of the eighteen cases examined is difficult to make. Our postpartum specimens were taken on the average about 8 days after delivery, to make certain of securing specimens before the patients left the hospital. We did not realize that the expected fall in pH had not yet taken place until after our study had been completed; otherwise, an effort would have been made to secure further specimens. It would seem logical that a little time might be required to readjust completely a disturbed acid-base equilibrium which had existed for several months.

Although the cases which we studied all showed hypertension and proteinuria, none of them gave abnormal figures for the blood urea (as shown in our tables (2), they were normal or the subnormal figures of pregnancy). The patients were, therefore, not suffering from serious impairment in renal function. No cases of eclampsia were included in our series. It seems quite obvious, as pointed out by Kydd, Oard, and Peters (1) and others, that there should be a fall in the blood pH as a result of eclamptic convulsions or severe renal complications. We have no pH observations covering this point, although very low figures for the CO<sub>2</sub> capacity of the blood in these conditions were obtained by Killian and Sherwin (22) in the senior author's laboratory a number of years ago. As shown by Wirz (13) the exertion of labor is sufficient to produce a marked temporary drop in the blood pH.

There are a number of analogies between the acid-base disturbance observed in pregnancy and the acid-base disturbance brought about by hyperventilation in such conditions as forced breathing, lowered O<sub>2</sub> tension, hot baths, and fever, for which conclusive evidence is available to show a shift in the acid-base balance towards the alkaline side. The condition here may be much more acute with a more rapid change in the acid-base balance.

In forced breathing Collip and Backus (23) noted a marked fall in the alveolar CO<sub>2</sub> tension and CO<sub>2</sub>-combining power of the blood with a decreased urinary acidity, while Grant and Goldman (24) found, in addition to the alveolar and blood CO<sub>2</sub> changes, a marked increase in the pH of both the blood and urine. In thirteen experiments the pH of the urine rose on an average from 5.2 to 7.4.

Schneider (25) has reported a decrease in the alveolar  $\text{CO}_2$  tension in the members of several expeditions to Pike's Peak. In the expedition to the Andes, Barcroft (26) reported a considerable increase in the blood pH of five members of the party, which results he obtained by the Dale-Evans colorimetric method, and a small increase with the gasometric method in another set of observations.

In his rather extensive study on hot baths with human subjects, Bazett (27) comments, regarding the respiratory changes: "A very marked hyperpnea gradually becoming more severe until it amounted to dyspnea, was seen in hot baths. The ventilation rate was increased enormously mostly by an increase in depth and only slightly by an increase in rate." He found in all experiments that the pH of the urine changed to the alkaline side and usually was between 6.8 and 7.5 even when the titratable acid excreted per hour was not decreased. The effect of high environmental temperatures upon the blood of dogs has been studied by Flinn and Scott (28). They found an increase in respiration which induced an uncompensated  $\text{CO}_2$  deficit with a marked rise in the blood pH.

The figures which Koehler (29) has reported for clinical fevers (chiefly influenza) correspond quite closely with many of those encountered in the last months of pregnancy; *i.e.*, they show a definite increase in blood pH (electrometric) with a decrease in  $\text{CO}_2$ .

#### CONCLUSION

The overwhelming mass of data now available, showing a fall in the blood  $\text{CO}_2$  and a rise in the blood pH during pregnancy, support the assumption that hyperventilation is one of the primary factors in the disturbed acid-base equilibrium. The preceding paper of Kydd, Oard, and Peters (1) presents no data to break down this hypothesis.

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## THE ALLEGED ALKALOSIS IN PREGNANCY

BY DAVID M. KYDD AND JOHN P. PETERS

*(From the Department of Internal Medicine of Yale University, New Haven)*

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The differences between Myers and his associates and the present authors, which have been raised in the preceding papers (1, 2) can be separated into two distinct points: (1) the effect of pregnancy on the pH of the blood; (2) the relation of the disturbance of pH to the reduction of serum bicarbonate, the existence of which is unquestioned.

Muntwyler, Limbach, Bill, and Myers (3) and Gaebler and Rosene (4) claim that the blood of pregnant women is more alkaline than that of normal women, while Kydd, Oard, and Peters (1) found the pH within normal limits. Myers, in his note (2), cites numerous other authorities in support of his claim. Anyone familiar with the literature will recognize that this matching of authorities may become an endless game. It is the very conflict of opinion and data in the literature that led Kydd, and presumably Myers, to reinvestigate the subject. The most significant characteristics of all the data in the literature are the extreme variability of the findings and the fact that the case for either high or low pH can, at best, be established only on a statistical basis.

How true this is of Myers' own figures is evident from Fig. 1, which illustrates the distribution of pH in normal subjects, normal pregnant women, and women with pregnancy toxemias. For normal subjects the authors have been forced to avail themselves of the data of Earle and Cullen (5), because they have been unable to find any series by Myers and his associates. As a substitute for such data the following statements are found:

"In a series of about twenty-five miscellaneous hospital cases, in which abnormal values for the pH were not anticipated, the figures varied between pH 7.35 and 7.43, with an average close to 7.39" (6). In the subsequent

article of Gaebler and Rosene (4) it is pointed out that "In the method as used in Professor Myers' laboratory at present, however, 5 per cent more indicator is added to the saline than before, to compensate for its dilution by the addition of 0.1 cc. of plasma to 2 cc. of saline. The results thus obtained for pH are more alkaline by 0.03 than by the method as originally

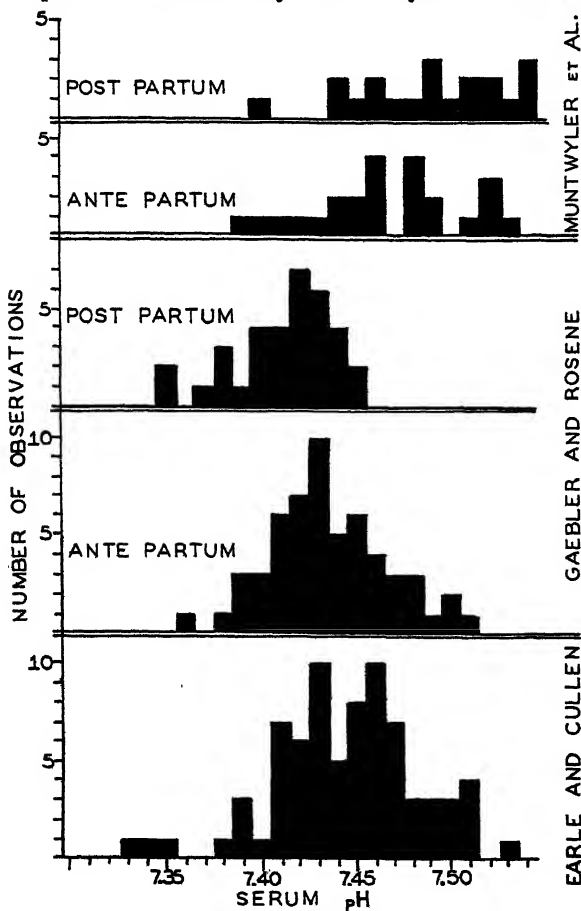


FIG. 1. The pH of serum in normal persons, pregnant women, and pregnancy toxemias. Abscissæ represent serum pH; ordinates, number of observations. The lowest section gives normal values reported by Earle and Cullen (5); the middle sections, values secured from pregnant women before and after delivery by Gaebler and Rosene (4); the top sections, similar data of Muntwyler, Limbach, Bill, and Myers (3) from patients with pregnancy toxemias.

described. . . . Introducing the correction of 0.03 mentioned above, the [normal] limits would be 7.38 to 7.46."

Data from such material are hardly satisfactory for the definition of normal limits. Nevertheless, it is worthy of note that the peak of the frequency chart (Fig. 1) of Gaebler and Rosene's analyses in normal pregnancy lies within the limits set by Myers for the non-pregnant normal individual. In fact, as the authors themselves admit, only 18 per cent of the blood samples obtained during pregnancy fell above the normal limit, 7.46. What is more significant is the fact that the distribution of both post-partum and antepartum groups of this series is quite similar to that of Earle and Cullen for normal individuals and exhibits not the least tendency to alkalosis. The abnormal pregnancy cases of Muntwyler, Limbach, Bill, and Myers (3) show much scattering with a greater frequency of high pH figures; but the cases are too few in proportion to the scattering to permit any importance to be attached to this point. There is, therefore, no convincing evidence of alkalosis in Myers' own data. The most that the most prejudiced critic could grant would be some tendency to greater scattering of values in pregnancy, with relatively more high than low pH values; and this only if the normal limits are to be defined by a restricted number of determinations on hospital patients, not normal subjects.

The respiratory studies cited by Myers (2) can be dismissed briefly. With low blood bicarbonate the  $\text{CO}_2$  tension must be reduced to maintain a normal pH and the respiratory volume must increase accordingly. The low alveolar  $\text{CO}_2$  and increased minute volume have, indeed, been adduced as evidence of acidosis. Against Bokelman and Rother's "convincing evidence that the pH of the urine is more alkaline during pregnancy than some time after delivery" (quotation from Myers, Muntwyler, and Bill (2)) might be placed the determinations of ammonia excretion so often erroneously cited as evidence of acidosis. However, both Myers and the authors, in any case, admit an abnormality of metabolism that results in a deficiency of base in serum and, presumably, throughout the body fluids. It is not unnatural to infer that the pregnant organism conserves base with less than the usual efficiency whatever may be the reason for such an abnormality.



Although there is a definite conflict of opinion in the literature about the reaction of the blood in pregnancy, there is one generalization that can be made concerning all the adequately controlled studies. Those who claim either acidosis or alkalosis, base their claims on semistatistical treatment of data. In none of them is high pH or low pH consistently found. In all series a large number of the determinations fall within normal limits.

Contrast with this the consistency of the bicarbonate reduction, illustrated in Fig. 2, again from Myers' own observations. There

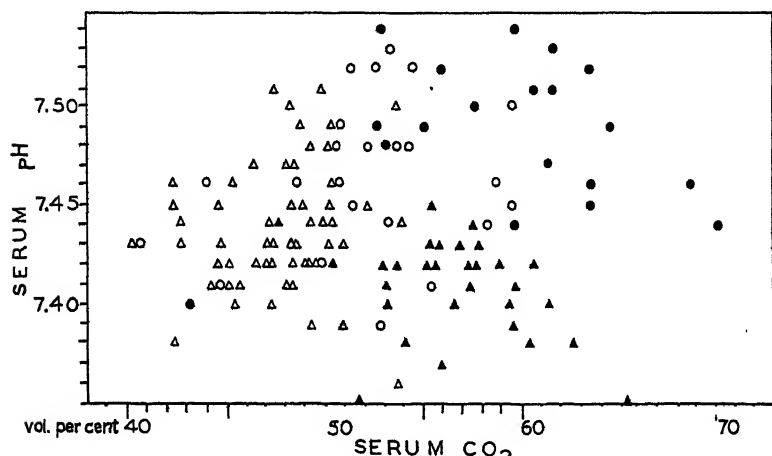


FIG. 2. The relation of serum pH to  $\text{CO}_2$  in pregnancy. The triangles represent observations on normal pregnant women by Gaebler and Rosene (4); the circles, similar observations in pregnancy toxemias, by Muntwyler, Limbach, Bill, and Myers (3). Solid triangles and circles indicate observations made after delivery.

can be no doubt of the reality of this disturbance nor of its direct relation to pregnancy. It is an invariable occurrence requiring no statistical treatment. By the same token there is in the literature no conflict of opinion or observation. The bicarbonate deficiency of pregnancy is an incontrovertible fact, as is the associated base deficiency. To associate as cause and effect such an inconstant variation of pH as even Myers admits and such a consistent deviation of bicarbonate is beyond the realm of statistics.

Overventilation affects primarily the ratio of free carbonic acid

to bicarbonate, causing the pH to rise; the bicarbonate reduction is to be regarded as a secondary, compensatory phenomenon. Gaebler and Rosene (4) found that the pH had returned to normal from 10 to 17 days post partum. Muntwyler, Limbach, Bill, and Myers (3) found the pH quite as high after delivery as before in their cases of abnormal pregnancy. Both agreed with Oard and Peters (1, 7) and others that both base and bicarbonate rose rapidly to normal after pregnancy. Again, Fig. 1 shows, in Gaebler and Rosene's figures, only a contraction of scattering without any change in the peak of the frequency data. Concerning these there is nothing further to add to the statement above: a questionably high pH in 18 per cent of cases cannot explain an indubitable bicarbonate deficit in 100 per cent. A still greater burden lies on Muntwyler and his associates when they attempt to ascribe the bicarbonate deficiency during pregnancy to over-ventilation, by implication placing high pH in the position of cause, and are then forced to confess that the bicarbonate deficiency disappears post partum, while its alleged cause still persists.

The authors sincerely regret that they have been forced to place so much emphasis on what may seem a minor point of difference with Myers, in view of the general agreement of their findings. However, they feel that it would be unfortunate to permit the introduction of a new misconception concerning the cause of bicarbonate and base deficits on which they confess their own investigations have thrown no light.

#### CONCLUSIONS

The data of Myers and his associates offer no satisfactory evidence that the pH of the serum in pregnancy is increased with any consistency nor that the well recognized reduction of bicarbonate results from overventilation.

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## THE ALLEGED ALKALOSIS IN PREGNANCY

A REPLY TO THE PAPER OF KYDD AND PETERS

BY VICTOR C. MYERS, EDWARD MUNTWYLER, AND ARTHUR H. BILL

*(From the Departments of Biochemistry and Obstetrics, School of Medicine, Western Reserve University and the Maternity Hospital, Cleveland)*

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The authors were given the opportunity to read the further comments of Kydd and Peters (1) under the title of "The alleged alkalosis in pregnancy" before publication. As noted in the literature references cited in our preceding paper (2), the great majority of workers who have determined the pH during pregnancy have observed a slight elevation and have interpreted this as supporting the hyperventilation hypothesis of the rise in blood pH and fall in blood bicarbonate. Since we have made similar observations, we have tentatively accepted this same hypothesis as explaining the facts observed. We are not irreparably committed to this hypothesis, and if Kydd and Peters can suggest a better one with facts to support it, we will be quite willing to accept it.

We do not believe that Kydd and Peters (1) can disregard the high pH observations recorded in the literature, which we have cited, by the statement: "Anyone familiar with the literature will recognize that this matching of authorities may become an endless game."

It is quite pointless for Kydd and Peters ((1) Figs. 1 and 2) to compare the pregnancy observations of Gaebler and Rosene (3) and Muntwyler, Limbach, Bill, and Myers (4) with the observations made on normal subjects by Earle and Cullen (5) in Nashville. In this series Earle and Cullen obtained pH values of 7.41 to 7.50 with an average of 7.45 on thirteen medical students. These normal values are higher than those generally reported and Earle and Cullen appear to have been at a loss to explain them, or reconcile them with the much larger series made in

Philadelphia by Cullen and Robinson (6). On twenty-seven normal subjects, Cullen and Robinson found the range to be between pH 7.28 and 7.41, although on twenty-one of these it lay between 7.35 and 7.40. Why did not Kydd and Peters (1) use these observations?

As the result of a large number of observations made on human plasma by both the electrometric and colorimetric methods, we feel that the normal pH generally falls between 7.35 and 7.45, with an average of 7.4. We regard pH 7.46 as the border-line of the abnormal, and pH 7.48 or above as fairly definite evidence of an uncompensated alkalosis. We were lead to this latter view as the result of an acid-base balance study on more than 150 blood specimens taken largely from peptic ulcer cases receiving alkali therapy (7). In contrast to the normal as defined above, Gaebler and Rosene (3) found the range of 56 antepartum findings in normal pregnancy to be from pH 7.35 to 7.51, with an average of 7.44, while Muntwyler, Limbach, Bill, and Myers (4) in their twenty-six cases of mild toxemias of pregnancy found their antepartum findings to range from 7.39 to 7.53 with an average of 7.47. A high pH is not invariably found during pregnancy, for the apparent reason that the acid-base disturbance is compensated in many cases, but the evidence is quite conclusive that the pH is above the normal limits in a considerable number of the cases.

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# THE URINARY EXCRETION OF INORGANIC PHOSPHATE IN FASTING WITH PARTICULAR REFERENCE TO THE EFFECT OF EXERCISE

BY ARTHUR G. MULDER, IRWIN E. PHILLIPS, AND MAURICE B.  
VISSCHER

*(From the Department of Physiology, University of Tennessee, Memphis,  
and the Department of Physiology, University of Illinois, Chicago)*

(Received for publication, July 18, 1932)

A series of carefully controlled fasts of from 5 to 7 days duration upon the authors as subjects provided opportunity for (1) a study of phosphate excretion during fasting and (2) a study of the influence of exercise upon the excretion of phosphates in the starving state.

Several previous observations have been made on phosphate excretion in fasting. Benedict (1915) summarized the earlier work and presented the findings in the 31 day fast of his subject L. Our observations were made primarily in order to study the effect of exercise, and therefore the first section of this paper dealing with the simple effect of starvation is reported as a confirmation, upon a number of subjects, of the earlier studies.

The influence of exercise upon phosphate excretion in the starving man is of interest because several observers, Embden and Grafe (1921), Hartmann (1924), Havard and Reay (1926), among others, have noted a rise in urinary phosphate excretion during and after exercise in the normal man. The reported observations have been made on subjects with such stores of carbohydrate as to make it conceivable that the excess metabolism was exclusively at the expense of that foodstuff. It seemed to us to be of importance to know whether in the starving subject in whom the metabolism of exercise is at the expense of body fat the situation might be different. If the intermediary metabolism of muscle with respect to the rôle of phosphorus differs in fat-burning and in carbohydrate-burning muscles it might be expected that the

starving man would not present an increased phosphate excretion in exercise.

At the time these experiments were performed the labile phosphorus in muscle was thought to be a hexosephosphate lactacidogen (Embden, 1927). Since the work of Eggleton and Eggleton (1928) and Fiske and Subbarow (1929), it is known that the larger share of the labile phosphorus is in the form of creatine phosphate. More recently Lundsgaard (1930) has brought forward evidence tending to show that creatine phosphate breakdown may yield the energy for contraction. There is a none the less important rôle to be played by a hexosephosphate in the anaerobic recovery process. In any case it is of importance to know whether the phosphate excretion in exercise is altered in the same direction and to the same extent in the starving as in the normal man.

### *Methods*

The general management of the starving subjects and the exercise carried out is described by Mulder, Phillips, and Visscher (1932). The method of collection of urine and general methods of analysis are likewise given there. The phosphate estimations were made by the method of Fiske and Subbarow (1925). The fecal phosphorus excretion was not studied because there was no defecation during the fasts after the 1st day.

### *Results*

#### *Influence of Starvation Itself*

In the four fasts of from 5 to 7 days duration included in this study, the phosphate excretion in general tended to increase with the progress of the fast. This will be observed in Tables I to IV in which the experimental observations are presented. Benedict (1915) observed the same phenomenon in the 1st week of L.'s fast, and it has been observed by some others, although it apparently does not occur when the exogenous protein catabolism was very high preceding the fast, as presumably was the case in S. A. B.'s fast of 7 days. In this connection it is worthy of notice that in Table I in the observations upon A. G. M. the phosphate excretion rise was less than in the other cases, and that the urinary nitrogen in this instance was high during the 1st days of starvation, indi-

cating a large protein breakdown probably due to a high protein intake just preceding the fast.

Several observers have paid attention to the source of the excreted phosphorus in starvation. In Benedict's (1915) summary it is pointed out that in calculating from the known phosphorus content of tissues and the observed nitrogen excretion there is invariably an excretion of phosphorus greater than would have come from the tissues burned. The reason for this excessive phosphate excretion can only be conjectured, but reasons will be brought forward to support the view that the excessive excretion is causally related to the ketosis and associated acidosis.

Benedict assumed for purposes of calculation that the ratio of nitrogen to phosphorus in the tissues burned for energy in the starving subject is 1:0.066. This figure approximates others in the literature. If it be taken as the basis for calculation in the experiments on fasting subjects reported here it is found that, for example, in I. E. P. in March, 1928, 26 per cent of the phosphate excreted is in excess of that which could have been derived from the burned tissue. On 1 day the excess was as great as 76 per cent of the amount derived from the tissue destruction. It is interesting to note that in all of our observations except upon A. G. M. the excess phosphorus excretion parallels the ketone excretion. In A. G. M. the height of ketosis reached was very low until the 5th day and is therefore not quite comparable with the other three experiments.

It appears to be plausible that the increased phosphate excretion with increasing ketonuria may be due to increasing acidosis. Fitz, Alsberg, and Henderson (1907) found that in mineral acid acidosis the excretion rate of phosphate was markedly elevated, bespeaking a disturbance of equilibrium by the displacement of the phosphate from its combination with calcium in the bone. Davies, Haldane, and Kennaway (1920) and Haldane, Hill, and Luck (1923) have noted that in experimental acidosis the rate of excretion of phosphate increases. Havard and Reay (1926) showed that the increase in urinary phosphate is closely paralleled by the phosphate rise in the blood. They consider it probable that the increased urinary excretion is due initially to the heightened blood level. Such a hypothesis is very attractive but it cannot be looked upon as a generalization covering other types of



TABLE I

*Excretion Studies on Subject A. G. M.*

March, 1928, body weight 72 kilos, height 178 cm. Last meal 13 hours before first period.

Time	State	Urine volume	Urine phosphate	Total urine N	P from metabolized protein calculated from N	Excess P actually excreted	Urine ketone body excretion
		cc.	gm. P per hr.	gm. per hr.	gm. per period	gm. per period	gm. per period
9 a.m.-10.30 p.m.	A.	690	0.033	0.561			
10.30 p.m.- 7 a.m.	R.	780	0.040	0.434			
Total, 22 hrs.....			0.784	11.26	0.771	+0.013	0.046
7 a.m.- 3 p.m.	A.	255	0.042	0.510			
3 p.m.-11 "	"	570	0.042	0.481			
11 " - 7 a.m.	R.	700	0.046	0.568			
Total, 24 hrs.....			1.038	12.47	0.823	+0.215	1.325
7 a.m.- 3 p.m.	A.	350	0.035	0.474			
3 p.m.-11 "	"	600	0.037	0.531			
11 " - 7 a.m.	R.	505	0.030	0.509			
Total, 24 hrs.....			0.812	12.11	0.799	+0.013	1.958
7 a.m.- 3 p.m.	A.	250	0.040	0.554			
3 p.m.- 7 "	E.	670	0.031	0.611			
7 " -11 "	A.	473	0.025	0.579			
11 " - 7 a.m.	R.	446	0.034	0.457			
Total, 24 hrs.....			0.819	12.85	0.848	-0.029	3.625
7 a.m.- 2.20 p.m.	A.	355	0.040	0.524			
2.20 p.m.- 6.20 "	E.	132	0.029	0.439			
6.20 " -10.20 "	A.	205	0.047	0.554			
10.20 " - 6.20 a.m.	R.	536	0.051	0.495			
Total, 23 hrs. and 20 min.....			0.999	11.88	0.784	+0.215	9.913
6.20 a.m.-12.20 p.m.	A.	316	0.040	0.498			
12.20 p.m.- 4.20 "	"	199	0.041	0.472			
Total, 10 hrs.....			0.402	4.88	0.322	+0.080	6.975

A. indicates activity; R., rest; E., exercise.

TABLE II

*Excretion Studies on Subject I. E. P.*

March, 1928, body weight 63 kilos, height 171 cm. Last meal immediately before first period.

Time	State	Urine volume	Urine phosphate	Total urine N	P from metabolized protein calculated from N	Excess P actually excreted	Urine ketone body excretion
		cc.	gm. P per hr.	gm. per hr.	gm. per period	gm. per period	gm. per period
11 p.m.- 7 a.m.	S.	180	0.035	0.229			
7 a.m.- 3 p.m.	A.	355	0.021	0.384			
3 p.m.-11 "	"	510	0.026	0.434			
11 " - 7 a.m.	S.	165	0.028	0.258			
Total, 24 hrs.....			0.591	8.60	0.568	+0.023	0.539
7 a.m.- 3 p.m.	A.	365	0.036	0.591			
3 p.m.-11 "	"	840	0.053	0.698			
11 " - 7 a.m.	S.	587	0.050	0.470			
Total, 24 hrs.....			1.114	14.07	0.931	+0.183	15.348
7 a.m.-11 a.m.	R.	340	0.065	0.722			
11 " - 3 p.m.	E.	420	0.042	0.632			
3 p.m.- 8 "	"	313	0.037	0.492			
8 " - 2 a.m.	S.	445	0.047	0.470			
2 a.m.- 7 "	"	247	0.054	0.432			
Total, 24 hrs.....			1.154	12.92	0.851	+0.303	14.750
7 a.m.- 3 p.m.	R.	665	0.061	0.573			
3 p.m.- 6 "	"	Lost					
6 " -11 "	"	455	0.070	0.452			
11 " - 7 a.m.	S.	510	0.051	0.295			
Total, 21 hrs.....			1.246	9.20	0.706	+0.540	16.800
7 a.m.- 3 p.m.	A.	505	0.055	0.365			
3 p.m.-11 "	"	507	0.036	0.433			
11 " - 7 a.m.	S.	268	0.030	0.292			
Total, 24 hrs.....			0.964	8.71	0.662	+0.302	10.392
" for fast.....			5.069	53.50			

A. indicates activity; S., sleep; R., rest; E., exercise.

TABLE III

*Excretion Studies on Subject M. B. V.*

December, 1928, body weight 81 kilos, height 184 cm. Last meal 28 hours before first period.

Time			State	Urine vol- ume	Urine phos- phate	Total urine N	P from metabo- lized protein calcu- lated from N	Excess P actually excreted	Urine ketone body excre- tion
				cc.	gm. P per hr.	gm. per hr.	gm. per period	gm. per period	gm. per period
11	p.m.- 7	a.m.	R.	125	0.027	0.266			
7	a.m.- 3	p.m.	A.	420	0.044	0.501			
3	p.m.-11	"	"	515	0.016	0.490			
11	" - 7	a.m.	R.	310	0.025	0.437			
Total, 24 hrs.....					0.680	11.42	0.754	+0.074	2.14
7	a.m.- 3.30	p.m.	A.	650	0.070	0.715			
3.30	p.m.-11	"	"	550	0.045	0.545			
11	" - 7	a.m.	R.	510	0.058	0.539			
Total, 24 hrs.....					1.397	14.48	0.956	+0.441	13.36
7	a.m.- 1	p.m.	A.	790	0.077	0.635			
1	p.m.- 3	"	a	100	0.056	0.434			
3	" - 5	"	b	132	0.053	0.418			
5	" - 7	"	E.	250	0.061	0.537			
7	" - 9	"	A.	370	0.051	0.629			
9	" -11.10	"	R.	320	0.059	0.548			
11.10	" - 7	a.m.	"	470	0.052	0.425			
Total, 24 hrs.....					1.438	13.36	0.816	+0.622	12.38
7	a.m.- 1	p.m.	A.	315	0.041	0.420			
1	p.m.- 3	"	"	160	0.053	0.447			
3	" - 5	"	"	70	0.048	0.362			
5	" - 7	"	"	70	0.042	0.404			
7	" - 9	"	"	270	0.041	0.506			
9	" -11	"	"	200	0.041	0.458			
11	" - 7	a.m.	R.	550	0.045	0.409			
Total, 24 hrs.....					1.056	10.15	0.670	+0.386	12.48

TABLE III—*Concluded*

Time			State	Urine vol- ume	Urine phos- phate	Total urine N	P from metabo- lized protein calcu- lated from N	Excess P actually excreted	Urine ketone body excre- tion
				cc.	gm. P per hr.	gm. per hr.	gm. per period	gm. per period	gm. per period
7	a.m.—	1 p.m.	A.	225	0.036	0.313			
1	p.m.—	3.15 "	"*	95	0.038	0.342			
3.15	" —	4.45 "	E.*	100	0.038	0.401			
4.45	" —	7 "	A.	95	0.045	0.397			
Total, 12 hrs.....					0.460	4.14	0.273	+0.187	5.34
Calculated for 24 hrs.....					0.920	8.28	0.546	+0.374	10.68
7	p.m.—	9 p.m.	A.†	100	0.055	0.452			
9	" —	11 "	A.	75	0.049	0.413			

A. indicates activity; R., rest; E., exercise; a, activity from 1.00 to 2.30 p.m., rest from 2.30 to 3.00 p.m.; b, rest from 3.00 to 3.45 p.m., exercise from 3.45 to 6.45 p.m.

\* The exercise was from 3.00 till 4.30 p.m.

† At 7.00 p.m. 6 gm. of glucose were eaten.

increase in elimination of phosphate, since the same authors have shown that in exercise in the normal subject, during which there is a very marked increase in phosphate output, there is only a small transitory rise in the blood phosphate, and the major part of the increased excretion occurs at a time when the blood level has fallen well below normal. It may be, of course, that the concentration of some fraction of the blood phosphates is determinative for the kidney excretion rate, and that this fraction is actually increased while the total quantity is decreased. Ultrafiltration studies have shown that about one-third of the blood phosphate is unable to pass through the pores of the collodion membrane and is, therefore, presumably not in simple ionic form. If phosphate is filtered through the glomeruli and reabsorbed in its passage through the tubules, as Schmitt and White (1928) have demonstrated to occur in the *Necturus* kidney, the ionic activity would be one of the important factors determining excretion rate. A change in the concentration of complex phosphates in the blood would, of course, alter the ionic activity of phosphate much less

TABLE IV

*Excretion Studies on Subject I. E. P.*

April, 1929, body weight 63 kilos, height 171 cm. Last meal  $\frac{1}{2}$  hour after beginning of first period.

Time			State	Urine volume	Urine phosphate	Total urine N	P from metabolized protein calculated from N	Excess P actually excreted	Urine ketone body excretion
				cc.	gm. P per hr.	gm. per hr.	gm. per period	gm. per period	gm. per period
7	a.m.-	3 p.m.	A.	975	0.042	0.668			
3	p.m.-12	"	"	765	0.025	0.420			
12	" - 8	a.m.	R.	98	0.027	0.192			
Total, 25 hrs.....					0.777	9.66	0.638	+0.139	0.02
8	a.m.-	4 p.m.	A.	180	0.031	0.420			
4	p.m.-12	"	"	250	0.040	0.549			
12	" - 8	a.m.	R.	220	0.037	0.518			
Total, 24 hrs.....					0.864	9.90	0.653	+0.211	2.39
8	a.m.-	4 p.m.	R.-A.	350	0.067	0.780			
4	p.m.-12	"	A.	435	0.064	0.742			
12	" - 8	a.m.	R.	415	0.050	0.556			
Total, 24 hrs.....					1.448	16.62	1.097	+0.351	5.99
8	a.m.-	4 p.m.	R.-A.	430	0.070	0.703			
4	p.m.-	4.30 "	E.	55	0.085	0.698			
4.30	" - 8	"	"	710	0.049	0.710			
8	" -12	"	A.	425	0.046	0.598			
12	" - 8	a.m.	R.	500	0.072	0.399			
Total, 24 hrs.....					1.534	13.94	0.920	+0.614	7.05
8	a.m.-	4 p.m.	A.	410	0.061	0.542			
4	p.m.-	8 "	"	205	0.073	0.532			
8	" -12	"	"	170	0.060	0.493			
12	" - 8	a.m.	R.	450	0.036	0.423			
Total, 24 hrs.....					1.308	11.82	0.780	+0.528	9.94
8	a.m.-	3.30 p.m.	R.-A.	290	0.036	0.493			
3.30	p.m.-	4 "	A.	31	0.056	0.616			
4	" -	4.30 "	E.	65	0.080	0.688			
4.30	" - 8	"	"	395	0.062	0.588			
8	" -11	"	R.	235	0.048	0.509			
Total, 15 hrs.....					0.769	7.94	0.524	+0.245	6.90
Estimated for 24 hrs.....					1.217	12.70			

A. indicates activity; R., rest; E., exercise; R.-A., rest first half of period, activity second half.

than would an equimolar change in the concentration of either the mono- or dibasic alkaline phosphates. It has not been possible to do so in these studies, but it would appear that an examination of the ionic activity of the phosphate in the blood in relation to the excretion rate of inorganic phosphate might yield valuable information.

It is well known that there is, in general, an increase in phosphate excretion in diabetes, as noted by Forbes and Keith (1914) in their compilation of the literature upon phosphorus metabolism. This increase is probably not a metabolic defect associated with the impaired carbohydrate burning, but is more apt to be a result of the disturbance in the buffer system in the body by the accumulation of the acid metabolites characteristic of the disorder. Diabetic acidosis would, according to this conception, be strictly analogous to starvation acidosis with respect to the increased phosphate excretion, the rise being occasioned simply by the general disturbance in the buffer system, and not because of any specific metabolic effect.

### *Influence of Exercise*

Seven experiments have been conducted with three adult male subjects during the four periods of fasting to determine the effect of exercise upon the rate of inorganic phosphate excretion. The results are given in Tables I to IV. In periods of from  $1\frac{1}{2}$  to 8 hours strenuous exercise the hourly excretion of phosphate in the urine is not increased above that in the rest periods immediately before or after the exercise. In four experiments there is, in fact, a more or less definite decrease in phosphate excretion with the exercise. These decreases are not consistent or large, and may be looked upon, for the present at least, as being of no significance. Comparing exercise periods of 1 day with corresponding periods in the following rest day, as in A. G. M., Table I, one observes no tendency to a higher level of excretion on the exercise days than on the rest days. In contrast to these findings are to be noted the consistent and rather large increases observed by Embden and Grafe (1921), Hartmann (1924), and Havard and Reay (1926) in their subjects who were not starving.

In two of the seven experiments the excretion during the exercise period was separated into that during the first 30 minutes

and that of the remainder of the exercise period. In these experiments it was found (see Table IV) that there is an initial increase in phosphate excretion in the early part of the exercise period followed by a fall to a level so low as to keep the average excretion rate during the whole period at a level no higher than that during rest. In the same subject (see Table II) in two 4 hour exercise periods the general average excretion rate was not elevated above that at rest. The observed early increase would seem to indicate that the character of the metabolism of phosphate in exercise is at least qualitatively the same in the starving as in the normal human. The magnitude and duration of the increase in phosphate excretion in exercise appears to be less in the starving than in the normal man. It appears unjustifiable, however, to deduce from the fact that over the whole of an exercise period in starvation there is no increase above the resting level of phosphate excretion, such as is found in the normal organism, that the metabolism of phosphorus-containing compounds is essentially different in the two cases. The starving organism is more economical in conserving its mineral stores than is the normal, as is illustrated by the well known fact that the urine in starvation becomes very poor in sodium chloride. The complicated mechanisms by which the kidney regulates the level of body constituents may be involved in the conservation of inorganic phosphate. Too little is known about the conditions governing excretion rate to justify any speculation as to the mechanism of the effect we have observed.

#### SUMMARY AND CONCLUSION

1. The urinary phosphate excretion during each of the four fasts reported exceeded that which is calculated to have been possible to derive from the protein metabolized. It is suggested that the acidosis caused the increased phosphate excretion.

2. In the seven exercise experiments performed the hourly excretion rate of urinary phosphate for the whole period of exercise was not increased above the general resting levels. This is in contrast to reported findings in unstarved subjects in whom the rate is markedly increased.

3. In the first 30 minutes of a period of exercise during starvation there was, in the two experiments in which the urinary excretion during exercise was subdivided into shorter intervals, a

definite increase in urinary phosphate excretion above the resting level.

4. No explanation is evident for the failure of the starving subject to maintain an increased rate of phosphate excretion in exercise.

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## STUDIES ON CRYSTALLINE INSULIN\*

### XVII. THE HYDROLYSIS PRODUCTS OF INSULIN

BY H. JENSEN AND OSKAR WINTERSTEINER

(From the Department of Pharmacology, the Johns Hopkins University, Baltimore, and the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York)

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In a recent communication (1), in which we described the isolation of glutamic acid from crystalline insulin, reference has been made to all the previous work on the hydrolysis products of insulin, and the reasons have been pointed out which induced us to take up the problem anew. Aside from glutamic acid, we were not able to add any new constituents to the list of amino acids reported in previous publications; nevertheless, we feel that a brief presentation of our observations in the more recent phase of this work will be of interest, since they supplement our findings concerning the composition of the insulin molecule, especially in relation to its quantitative composition. We also believe that the problem is of sufficient importance to justify the inclusion of results which are of essentially negative character.

4 gm. of crystalline insulin were hydrolyzed by boiling with 40 cc. of 25 per cent sulfuric acid for 25 hours. The solution was neutralized with barium hydroxide. The filtrate, after thorough washing of the barium sulfate, was concentrated *in vacuo* at slightly alkaline reaction in order to remove ammonia. Cystine and tyrosine separated during this concentration. These amino acids were extracted with butyl alcohol *in vacuo* according to Dakin's procedure (2), and were identified by analysis. The filtrate from the cystine and tyrosine was then extracted with butyl alcohol for 30 hours in a small all-glass Dakin extractor of about 20 cc. capacity, which had also been used for the separation of

\* An investigation carried out under grants from the Carnegie Corporation of New York and the Chemical Foundation.

cystine and tyrosine. More cystine was thrown out of the aqueous solution during the extraction. The basic amino acids contained in the aqueous phase were precipitated with phosphotungstic acid in order to effect a separation from the dicarboxylic acids, and the latter were converted into their barium salts in the customary manner. Only glutamic acid could be identified in this fraction, as described in a previous paper (1). Attempts to isolate aspartic acid as the lead or copper salt failed. Although precipitates were obtained by the appropriate procedures, these were shown to contain mainly cystine as the organic component. The filtrate from the insoluble barium salts, in which we hoped to encounter glycine, was freed of barium with sulfuric acid and brought to dryness. No evidence, however, for the presence of glycine in this fraction could be obtained. The semicrystalline material secured from the residue by fractional precipitation with alcohol showed a melting point far above that recorded for glycine. Elementary analysis of this material gave figures which agreed well with those calculated for leucine. Treatment with potassium cyanate yielded a ureido compound melting at 204°, which is in fair agreement with the melting point found by us for the ureido compound from authentic leucine (208°). The melting point of the ureido compound of glycine is reported as 171–173°. Apparently the leucine, which is present in insulin in comparatively large amounts, had not been completely extracted by the butyl alcohol in the Dakin procedure.

The separation of the *hexone bases* in the phosphotungstate precipitate was effected by the method of Vickery and Leavenworth (3) as recently modified by Calvery (4). Arginine and histidine were isolated in the form of the corresponding flavianates, and lysine was identified as the picrate. The histidine fraction was found to be contaminated with cystine, and was freed from it by precipitating the latter as the copper salt, according to the method of Vickery and Leavenworth (5), who also frequently encountered cystine in the histidine fraction.

The *monoamino acid fraction*, after evaporation of the butyl alcohol, was taken up in 15 cc. of water and the solution allowed to stand in the refrigerator for several days. A precipitate formed, which consisted mainly of tyrosine. The filtrate was taken to dryness and the residue extracted with absolute alcohol. The

material soluble in alcohol was further purified by evaporation and reextraction with alcohol. The alcohol-soluble part was again extracted with water and the whole procedure repeated several times, the material remaining insoluble in water and in alcohol being discarded. The final product, which should contain proline, was easily soluble in absolute alcohol and in water. A considerable percentage of the total nitrogen of this fraction was found to be present as non-amino nitrogen. However, several attempts to secure crystalline derivatives of proline from this fraction met with failure. Since we agree with other investigators that the presence of non-amino nitrogen in the monoamino acid fraction of a protein hydrolysate is not sufficient proof for the presence of proline or oxyproline, the question whether these amino acids occur in insulin must be left open for the present. In a previous communication (1) we suggested the possibility that a part of the glutamic acid is converted into pyrrolidonecarboxylic acid during the operations following the hydrolysis. This acid possesses solubility properties similar to those of proline and should therefore be found in the proline fraction. It may be mentioned that this fraction gave the color reactions described by Dakin (2) as characteristic of  $\beta$ -hydroxyglutamic acid, but according to Harington and Randall (6) it is very doubtful whether these tests can be considered as specific for that compound.

In the alcohol-insoluble part of the monoamino acid fraction all the nitrogen was found to be present as amino nitrogen. This fraction was extracted with absolute methyl alcohol in the hope of encountering valine in the extract. We found, however, that leucine is also soluble in methyl alcohol to a certain extent. The portion soluble in methyl alcohol was therefore subjected to the fractionation described by Brazier (7), based upon the different solubilities of the copper salts. The copper salts insoluble in water contained leucine only. The solution of the water-soluble copper salts was evaporated to dryness and the residue stirred up with anhydrous acetone to remove the last traces of moisture. The dry copper salts were extracted three times with absolute methyl alcohol. The copper was removed from the soluble fraction with hydrogen sulfide, and the filtrate from the copper sulfide taken to dryness. A semicrystalline product was obtained from the solution of this residue in dilute ethyl alcohol, but analysis

indicated that it consisted of a mixture. The presence of valine or oxyvaline in insulin must therefore still be considered as doubtful. It might be pointed out that the copper salt of isoleucine is also soluble in methyl alcohol and that the analytical data did not exclude the possible presence of this amino acid.

The remaining portion of the monoamino acid fraction, insoluble in absolute methyl and ethyl alcohols, weighed about 1.3 gm. and was found to consist mainly of leucine, contaminated with some tyrosine. Colorimetric determination of the tyrosine content, according to the method of Folin and Looney (8), indicated that between 80 and 100 mg. were present. The method of Habermann and Ehrenfeld (9) for the separation of tyrosine and leucine was tried on this fraction but did not afford a quantitative removal of the tyrosine. Furthermore, an attempt was made to fractionate the material by precipitation of the aqueous solution with ethyl alcohol. Various fractions were obtained which, however, did not differ greatly in their analytical composition. The carbon and nitrogen content agreed fairly well with the figures calculated for leucine, although the hydrogen content was generally lower (8 to 9, instead of 10.0 per cent). Treatment of these various fractions with potassium cyanate yielded the ureido compound of leucine only, for which we found a melting point of 208° (205°, according to Lippich (10)). This fact, as well as the analytical results, seems to preclude the presence of appreciable quantities of glycine, alanine, serine, glutamic acid, and phenylalanine. Analytically pure leucine could be obtained from this fraction by fractional crystallization and by precipitation as the copper salt. On the basis of this evidence we are inclined to assume that the remaining 1.2 gm. of this fraction (after subtraction of the tyrosine content) were composed principally of leucine. When the small amount of leucine found in the aqueous phase after extraction with butyl alcohol (glycine fraction) and the small quantities probably extracted with methyl alcohol are taken into account, a figure of about 30 per cent of leucine in insulin would represent a conservative estimate.

#### DISCUSSION AND SUMMARY

The work reported in the experimental part on the hydrolysis of 4 gm. of crystalline insulin substantiates previous findings in

regard to the presence of cystine, tyrosine, arginine, histidine, and leucine. The presence of lysine has been established by the analysis of the picrate. We have already reported the isolation of glutamic acid as a new constituent. We are still in doubt as to the presence of proline and valine. Leucine accounts for a comparatively large part of the molecule. No evidence of the occurrence of aspartic acid, hydroxyglutamic acid, glycine, or of any constituent foreign to the protein molecule has been obtained. In view of the great difficulties necessarily involved in the separation of such small quantities of amino

TABLE I  
*Distribution of Amino Acids in Crystalline Insulin*

Amino acid	Amount present	Method of determination
	<i>per cent</i>	
Tyrosine.....	12	Colorimetrically, Folin-Looney
Cystine.....	12	“ “ or calculated from total S content; Sullivan's method gives 8 per cent
Glutamic acid.....	21	Calculated from amide nitrogen of Van Slyke N distribution
Leucine.....	30	Isolated as such
Arginine.....	3	Calculated from Van Slyke N distribution
Histidine.....	8	“ “
Lysine.....	2	“ “
Total.....	88	

acids, we do not wish to contend, of course, that our analysis gives sufficient proof that other amino acids or constituents of unknown nature do not exist in the insulin molecule, but we hold it improbable that such constituents occur there in very large amounts. In order to support this statement we give in Table I a tentative calculation of the distribution of amino acids, admitting the disputability of our assumption that all of the amide nitrogen is derived from glutamic acid, and the uncertainty attached to the figures for the hexone bases, which were determined by the method of Van Slyke. The percentage figures are not corrected for the amount of water taken up during the hydrolysis.

*Analytical Data**Tyrosine*

$C_9H_{11}O_3N$ . Calculated. C 59.60, H 6.12, N 7.74  
 Found. " 59.20, " 5.91, " 7.54

*Cystine*

$C_6H_{12}O_4N_2S_2$ . Calculated. S 26.67, N 11.65  
 Found. " 26.31, " 11.39

*Arginine Flavivanate*—Decomposed at 270°; dried at 105°.

$C_6H_{14}O_2N_4 \cdot C_{10}H_6O_6N_2S$ . Calculated. S 6.56  
 Found. " 6.58, 6.32, 6.37

*Histidine Diflavivanate*<sup>1</sup>—Decomposed with blackening and effervescence at 248°; dried at 105°.

$C_6H_9O_2N_3 \cdot 2C_{10}H_6O_6N_2S$ . Calculated. S 8.19, N 12.52  
 Found. " 8.80, " 12.30, 12.11

*Lysine Picrate*—Decomposed with slight explosion above 250°; dried at 105°.

$C_6H_{14}O_2N_2 \cdot C_6H_5(NO_2)_3OH$ . Calculated. C 38.38, H 4.57, N 18.66  
 Found. " 38.62, " 4.63, " 17.67  
 " 38.21, " 4.52

*Leucine (Purest Fraction, m.p. 288°)*

$C_6H_{13}O_2N$ . Calculated. C 54.87, H 10.00, N 10.69  
 Found. " 55.20, " 10.15, " 10.60

*Ureido Compound of Leucine, m.p. 208°*

$C_7H_{14}O_3N_2$ . Calculated. C 48.24, H 8.10, N 16.09  
 Found. " 48.27, " 7.77, " 15.71

*Leucine Fraction (Impure, Contains Tyrosine)*

Found. C 53.88, H 8.01, N 10.41, amino N 10.12, ash 0.72

*Mixture of Leucine and Tyrosine, Extracted with Absolute Methyl Alcohol and Ethyl Alcohol*

Found. C 54.53, H 8.44, N 10.55, amino N 10.08, ash 1.03

*Leucine Fraction, Crystalline, m.p. 285°, First Precipitate with Alcohol*

Found. C 54.10, H 9.44, N 10.43, ash 1.80

*Leucine Fraction, Crystalline, m.p. 280°, Second Precipitate with Alcohol*

Found. C 54.28, 54.36; H 9.10, 9.10; N 10.49; ash 1.0, 0.35

*Leucine Fraction, Filtrate from Crystalline Fractions Obtained with Alcohol*

Found. C 54.59, 54.60; H 7.88, 7.70; N 10.29; ash 0.54, 0.87

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<sup>1</sup> The monoflavivanate sintered at about 190° and decomposed at 212°. In agreement with Vickery (11), we found this salt unsuitable for analysis.

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## IRON AND THORIUM PRECIPITATION OF BIOLOGICAL FLUIDS FOR SUGAR AND OTHER ANALYSES

BY ALEXANDER STEINER, FRANK URBAN, AND EDWARD S. WEST  
(*From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis*)

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West, Scharles, and Peterson (1) have used  $\text{HgSO}_4\text{-BaCO}_3$  precipitation in the preparation of blood filtrates for true sugar determination by the Shaffer-Hartmann method. In this procedure the precipitating agents are removed along with non-sugar materials and the filtrates are relatively free from added electrolytes. Due to the time required for the preparation of filtrates as well as the cost of mercuric sulfate, the method is not well suited to routine use. For a number of years one of the writers (W.) has been attempting to find a metallic sulfate other than mercury which could be used with  $\text{BaCO}_3$  in a similar way, but would be free of these objections. During this search it was found that ferric sulfate serves admirably for the preparation of filtrates of blood, plasma, spinal fluid, and milk. These filtrates, while containing much more nitrogen than those prepared with mercury, give the same sugar values by the Shaffer-Hartmann method. Iron cannot, however, be satisfactorily substituted for mercury in the treatment of urine and hydrolyzed tissues.

It has been found by another of the writers (U.) that thorium sulfate may be used similarly with  $\text{BaCO}_3$  for the preparation of filtrates.

Michaelis (2) used colloidal ferric hydroxide and heat for deproteinization as did Shaffer (3), and Somogyi (4) has prepared blood filtrates by treatment with ferric sulfate followed by neutralization with sodium hydroxide. Somogyi's method does not completely remove the iron and for accurate sugar values the filtrates must be further treated with sodium carbonate, necessitating a second filtration. By his method it is also necessary to adjust the concentration of the sodium hydroxide solution so that the pH of the

mixture is optimum for precipitation. By using solid barium carbonate to neutralize the ferric sulfate, as proposed in this paper, the pH is automatically adjusted, both iron<sup>1</sup> and sulfate are removed, and the filtrates give true sugar values, by the Shaffer-Hartmann method, without further treatment. The precipitations filter rapidly and the method is well suited to routine determination of true blood sugar (by the Shaffer-Hartmann reagent) from the standpoint of simplicity, accuracy, and cost. The method yields filtrates which are practically free from added salts.

An especial advantage of the method is that it may be applied in unchanged form to biological fluids containing variable and smaller quantities of protein than blood, such as plasma, spinal fluid, and milk. All that is necessary in the  $\text{Fe}_2(\text{SO}_4)_3\text{-BaCO}_3$  technique is to use an excess of  $\text{Fe}_2(\text{SO}_4)_3$  which is removed in the precipitation.

#### EXPERIMENTAL

Both the macro and micro iron procedures for blood will be described, as well as the thorium method. The latter requires a little more time and is somewhat expensive, yet may be of value for certain purposes.

#### *Reagents*

*Ferric Sulfate*—This is approximately 17 per cent  $\text{Fe}_2(\text{SO}_4)_3$  by volume. Our reagent was prepared from Mallinckrodt's c.p. ferric sulfate + aqua (20 to 22 per cent  $\text{H}_2\text{O}$  according to the manufacturers) by dissolving 21 gm. in  $\text{H}_2\text{O}$  and diluting to 100 cc. Anhydrous ferric sulfate does not dissolve well.

*Barium Carbonate*—Mallinckrodt's technical precipitated barium carbonate. For the thorium precipitation a c.p. grade should be used, due to the presence of sulfide in the technical grade which thorium does not remove.

*Thorium Sulfate*—c.p., obtained from Eimer and Amend.

*Sugar Reagent*—Sugars were determined by a Shaffer-Hartmann reagent of the following composition in gm. per liter: anhydrous  $\text{Na}_2\text{CO}_3$ , 25.0;  $\text{NaHCO}_3$ , 20.0; Rochelle salt, 25.0;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 7.5; KI, 1.0;  $\text{KIO}_3$ , 20.0 cc. of 1 N solution (35.66 gm. of  $\text{KIO}_3$  per liter).

<sup>1</sup> Iron determinations on eight successive filtrates by the sulfocyanate method showed less than 0.13 mg. per 100 cc. of filtrate. This represents less than 0.0065 mg. of iron in the 5 cc. used for sugar analysis.

*Iron**Macro Method*

5 cc. of blood are laked in 50 cc. of  $H_2O$  in a 125 cc. flask. 5 cc. of ferric sulfate solution are added with mixing. About 7 gm. of  $BaCO_3$  are then added and the mixture whirled around for a few seconds until most of the  $CO_2$  has escaped. The flask is stoppered, shaken vigorously, and pressure released. This is repeated until no pressure is developed. At this stage the liquid should not redden blue litmus. If it does, add a pinch of  $BaCO_3$  and shake again. Filter and determine sugar in the usual way. The neutralization and filtration proceed rapidly. A trace of barium remains in the filtrates which, however, in no way interferes with the sugar determination by the Shaffer-Hartmann method. For other sugar reagents it may be desirable to remove this with a drop of saturated  $Na_2SO_4$  solution or of  $H_2SO_4$ . This point has not been investigated. In calculating results it must be remembered that the dilution is 1:12.

*Micro Method*

0.2 cc. of blood are laked in 7.6 cc. of  $H_2O$  in a 15 cc. centrifuge tube. 0.2 cc. of ferric sulfate solution are added and mixed with a thin rod. Approximately 0.3 to 0.5 gm. of  $BaCO_3$  are then stirred in, and the tube closed with the thumb and given a few vigorous shakes. The precipitate is centrifuged down and the supernatant liquid filtered through a small rapid filter (about 3 cm. diameter). 5 cc. of the filtrate are taken for the sugar determination. Results by this procedure agree well with those by the macro method.

Milk is diluted 1:20 and then precipitated as in the macro method for blood.

Plasma filtrates are prepared in the same manner as blood filtrates.

Spinal fluids may be treated as follows: 2 cc. of the fluid are added to 7 cc. of  $H_2O$ , followed by 1 cc. of ferric sulfate solution with mixing. 2 to 3 gm. of  $BaCO_3$  are then added, and neutralization and filtration carried out in the usual way.

*Procedure with Thorium*

This has been used only in the precipitation of blood and plasma at a dilution of 1:10, but would undoubtedly work in other cases.

5 cc. of blood are laked in 45 cc. of  $H_2O$  in a 125 cc. flask. 1 gm. of solid  $Th(SO_4)_2$  is added and the mixture shaken occasionally during 15 minutes. 1 gm. of pure  $BaCO_3$  is then added and neutralization and filtration carried out as in the iron method. The filtrates are quite free of thorium and sulfate and give the same sugar values as do the iron filtrates.

## DISCUSSION

The sugar content of a number of human and animal bloods and of plasma, spinal fluid, and milk has been determined. Simul-

TABLE I

*Comparative Sugar Determinations Showing That Blood and Other Filtrates Prepared by Deproteinization with  $Fe_2(SO_4)_3$ - $BaCO_3$  Give True Sugar Values*

Experiment No.	Samples	Iron	Mercury
		mg. per cent	mg. per cent
1	Human blood	73	72
2	" "	119	119
3	" "	86	86
4	Beef "	64	66
5	Pig "	50	50
6	Sheep "	38	39
7	Plasma	100	100
8	"	82	83
9	Spinal fluid*	88	90
10	Milk	4200	4200

\* Dilution of filtrate 1:5.

taneous determinations on  $HgSO_4$ - $BaCO_3$  filtrates were run as controls. Table I summarizes some of the results obtained on iron filtrates as well as those on mercury filtrates for comparison. Agreement is good.

Non-protein nitrogen values<sup>2</sup> on iron and thorium filtrates of blood are practically identical, but less than those of tungstate filtrates. The following series of figures represent, in order, values obtained on iron, thorium, and tungstate filtrates of five

<sup>2</sup> Before determination of non-protein nitrogen it is necessary to precipitate the trace of barium with a drop of  $H_2SO_4$  or saturated  $Na_2SO_4$  and filter.

human bloods: (1) 28, 29, 36; (2) 39, 39, 49; (3) 32, 29, 39; (4) 23, 24, 33; (5) 105, —, 143. If the non-protein nitrogen values of the iron and thorium filtrates are increased by 30 per cent, figures very near those of tungstate filtrates are obtained.

Iron and thorium blood filtrates contain all of the urea and creatinine and may be used for determining these substances after removal of the trace of barium with a drop of saturated  $\text{Na}_2\text{SO}_4$  and filtration. A considerable proportion of the uric acid is absent from these filtrates.

#### SUMMARY

Methods for preparing filtrates of blood, plasma, spinal fluid, and milk by precipitation with ferric sulfate-barium carbonate and thorium sulfate-barium carbonate are described.

Such filtrates are easily prepared and give true sugar values by the Shaffer-Hartmann method.

The filtrates may be used for the determination of creatinine and urea. Non-protein nitrogen values are about 25 per cent lower than those found on tungstate filtrates.

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## THE RACEMIZATION OF AMINO ACIDS IN AQUEOUS SOLUTION BY ACETIC ANHYDRIDE

By VINCENT DU VIGNEAUD AND CURTIS E. MEYER

*(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)*

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In a previous investigation du Vigneaud and Sealock (1) have reported that the sodium salt of acetyl-*L*-tryptophane in aqueous solution at 35–40° is completely racemized by acetic anhydride within a few hours. The ease of racemization and particularly the mild conditions under which the reaction occurred seemed to us to offer an excellent method for racemizing amino acids if the reaction should prove to be a general one. The present investigation was therefore undertaken to extend the study to other representative amino acids and further to study in greater detail the conditions under which the reaction will take place.

The amino acids selected for the work were methionine and cystine, the sulfur-containing amino acids, glutamic acid representing the dibasic acids, arginine as an example of the basic amino acids, tyrosine and phenylalanine as representatives of the monoamino acids, and finally proline which contains a secondary amino or imino group.

The sodium salts of the acetyl derivatives of all these amino acids with the exception of proline were racemized in aqueous solution by acetic anhydride. In the case of proline no racemization occurred. With cystine some decomposition resulted with the separation of free sulfur, although the reaction was carried out at only 36°. When free acetyl-*L*-cystine in aqueous solution without any NaOH was treated with acetic anhydride, no racemization occurred, nor was there any decomposition. Furthermore, the acetyl-*L*-cystine was not racemized or decomposed by the alkali alone under the above conditions.

The lack of racemization of the proline and the splitting out of sulfur from cystine during its racemization we regard as being very



significant from the standpoint of the mechanism of the reaction. This question of the mechanism will be dealt with more fully in a later publication.

In order to obtain some idea of the rate of racemization, a large amount of acetylglutamic acid was subjected to the racemization procedure, and, at stated intervals, samples were removed and the decrease in optical activity determined. Since the rotation of acetyl-*d*-glutamic acid is rather small and therefore not suitable for showing small differences in rotation, the samples were hydrolyzed and the glutamic acid separated as the hydrochloride. The free glutamic acid was then isolated and dissolved in 1 equivalent of hydrochloric acid for the determination of the rotation. The change of rotation with respect to time is shown in Chart I. Within 15 minutes the material was 50 per cent racemized, showing that the racemization proceeded very rapidly at first. After that the rate of the reaction gradually decreased. At 8 hours the material isolated was completely racemized. It is possible, of course, that a small amount of active material might have still remained at the 8 hour point and in the isolation some fractionation might have occurred. The yields obtained in the isolations, however, precluded the possibility of this affecting significantly the results. Furthermore, an experiment was run to test the possibility of fractionation by taking a mixture of 0.10 gm. of *d*-glutamic acid ( $[\alpha]_D^{25} = +30.52^\circ$ ) and 0.90 gm. of *dl*-glutamic acid. The hydrochloride was isolated and the free glutamic acid then prepared as in the original experiment. The glutamic acid isolated had a rotation of  $[\alpha]_D^{27} = +3.10^\circ$ , which shows that practically no fractionation occurred.

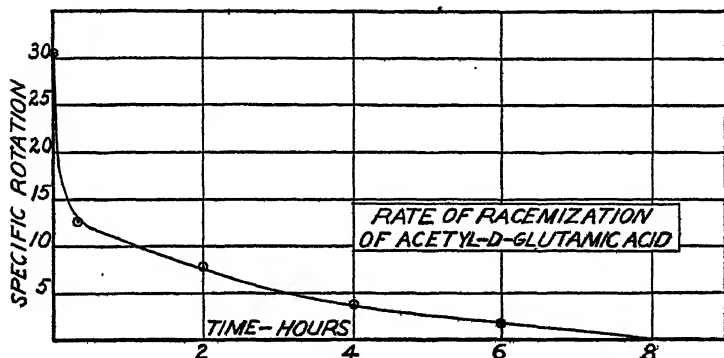
The ease of racemization differed somewhat with the various amino acids. For example, during the same time interval that led to a complete racemization of tryptophane, only partial racemization of acetylglutamic acid resulted.

It was of some importance from a preparative as well as a theoretical standpoint to determine whether or not the racemization was confined to the acetyl derivatives of the amino acids. The formyl derivative of *l*-phenylalanine was therefore tried. Racemization occurred, although not as readily as in the case of the acetyl derivative. For instance the acetyl compound was completely racemized within 24 hours, and probably much sooner, whereas the

formyl compound which originally had a specific rotation of  $+75^\circ$  still showed a rotation of  $+17^\circ$  at 24 hours. It was necessary to treat the material for 48 hours longer to racemize it completely. This work had been carried out at  $36^\circ$ . At a higher temperature racemization occurs of course much more rapidly. At  $76^\circ$  the racemization was complete within 12 hours.

The fact that racemization can be obtained with the formyl derivative makes possible its direct racemization, where the inactive formyl compound is desired for resolution purposes.

We have repeatedly tried to racemize the free acetylamino acid instead of the sodium salt in aqueous solution with acetic anhydride. Only slight racemization, if any, occurred. We found no



· CHART I. The racemization of amino acids in aqueous solution by acetic anhydride.

racemization with acetylglutamic acid, and in the case of acetylphenylalanine only a relatively slight decrease in rotation resulted from 120 hours action, whereas the sodium salt was completely racemized within 24 hours. It is also very interesting that Behr and Clarke (2) obtained very little racemization when they acetylated tyrosine by adding acetic anhydride to a suspension of tyrosine in water at  $90-95^\circ$ . This lack of racemization of the free acetylamino acid and the fact that racemization of the sodium salt takes place in the presence of water are quite in contrast to the catalytic racemization of Bergmann and Zervas (3) in which the free acetylamino acid dissolved in glacial acetic acid is racemized by acetic anhydride in the complete absence of water.

Although the sodium salts of the acetyl amino acids in water have an alkaline reaction towards litmus, the reaction mixture becomes acid to litmus immediately upon the addition of acetic anhydride. Since we have shown that the racemization is a time reaction, it is apparent that the racemization must take place in acid solution. It should follow from this that the racemization should take place about as readily for a mixture of the free acetyl amino acid and an equivalent of sodium acetate as for the sodium salt of the acetyl amino acid. This was shown by experiment to be the case, acetyl-*l*-phenylalanine being used.

The greater ease of the racemization of the sodium salt in contrast to the free acid might be due either to a specific effect of the sodium ion or to too great an acidity which might prevent or hinder the racemization. To test the former possibility, racemization of the free acid was attempted in the presence of 1 mol of NaCl in place of the sodium acetate. Very little racemization resulted, which showed that the reaction was not due to some catalytic effect of the sodium ion. To test whether the reaction was dependent on the hydrogen ion concentration the sodium acetate experiment was repeated but 1 mol of sulfuric acid was added. There was only a small decrease in rotation. Finally, to rule out entirely the question of the sodium ion and to test whether a buffer other than sodium acetate might work, racemization was carried out with 1 mol of pyridine in place of the sodium hydroxide. Complete racemization resulted within 24 hours. Furthermore, since monoacetyl-*d*-arginine is not as acidic in reaction as the acetyl derivatives of the monoamino acids or the dibasic acids, it occurred to us that it should be capable of racemization without any other base present. As was expected, complete racemization occurred.

N-Acetyl-*l*-tyrosine was prepared in excellent yield by acetylating an alkaline solution of tyrosine with acetic anhydride. The compound crystallized in rod-like crystals from water. The Milon reaction was positive and the analytical data indicated a monoacetyl derivative. The melting point of 152–154° (corrected) is somewhat higher than that reported by Takenaka (4) which was 146–148° for monoacetyl-*l*-tyrosine prepared from the ethyl ester of acetyl-*l*-tyrosine. The specific rotation of our product  $[\alpha]_D^{25} = +47.50^\circ$  for a 2 per cent aqueous solution agrees well with that found by Behr and Clarke (2), which was 46.2° for a 4.4 per cent

solution in water for their acetyl-*l*-tyrosine prepared by the addition of acetic anhydride to an aqueous suspension of tyrosine. By a procedure very similar to the one we have employed Bergmann and Zervas (3) obtained diacetyl-*l*-tyrosine, which crystallized in needles and melted at 170°.

When tyrosine was acetylated under the conditions necessary to bring about racemization we obtained the diacetylated inactive tyrosine. The difference between this and the above procedure was that in the racemization reaction we employed much more acetic anhydride and less sodium hydroxide. The diacetyl-*dl*-tyrosine crystallized in tufts of small needles, melted at 170° (corrected), and gave a negative Millon reaction.

An observation made in our racemization work on phenylalanine which might be significant from a metabolic standpoint is worthy of attention. The acetyl derivative of our *d*-phenylalanine had a negative rotation of  $[\alpha]_D = -51^\circ$  while Knoop and Blanco (5) reported practically the same negative rotation for the acetyl derivative of naturally occurring *l*-phenylalanine. After the feeding of inactive acetylphenylalanine to a dog, the latter investigators isolated from the urine an acetylphenylalanine having a levorotation. They came to the conclusion that the body could oxidize the acetyl derivative of the unnatural form more readily than that of the natural enantiomorph. These investigators did not report the isolation of the free phenylalanine from the acetyl derivative, although they did prepare the acetyl derivative of an active phenylalanine which they received from H. Fischer and Schoeller. This acetyl derivative they reported as having a rotation of  $[\alpha]_D = -51.8^\circ$ , but likewise they do not state the rotation of this free phenylalanine. It must follow that the acetyl derivative isolated from the urine was predominantly that of *d*-phenylalanine and that the body oxidizes more readily the acetyl derivative of the naturally occurring form, in contrast to the conclusion of Knoop and Blanco.

We hope to study this question further by the feeding of the acetyl derivatives of both the *l*- and *d*-phenylalanines.

It might also be pointed out that the acetyl derivative of the racemized naturally occurring methionine was identical in physical properties with the acetyl derivative of the racemic methionine synthesized by the method of Windus and Marvel (6).

## EXPERIMENTAL

*Preparation of Acetyl-dl-Methionine*

The *dl*-methionine was prepared by the method of Windus and Marvel (6). To an ice-cold solution of 5 gm. of *dl*-methionine in 10 cc. of water and 16.75 cc. of 2 N NaOH, 10 cc. of 2 N NaOH and 1 cc. of acetic anhydride were added. The solution was kept cold in an ice bath and, during the addition of the acetic anhydride, it was shaken vigorously. Eight such additions were made at 2 minute intervals. After the solution had stood at room temperature for 20 minutes an amount of 6 N  $\text{H}_2\text{SO}_4$  corresponding to the NaOH used was added.

After removal of the water and acetic acid by vacuum distillation, the acetylmethionine was extracted with hot absolute ethyl acetate. After evaporation of the ethyl acetate, the compound was recrystallized from the minimum amount of ethyl acetate, and 5.2 gm. of beautiful prismatic needle-like crystals were obtained. The product melted at 114–115° (corrected). The acetylmethionine can also be crystallized from water.

*Analysis*

3.578 mg. substance: 0.226 cc. N at 24.5° and 751 mm.

$\text{C}_7\text{H}_{13}\text{O}_2\text{NS}$ . Calculated, N 7.32; found, N 7.16

*Preparation of Acetyl-l-Methionine*

The *l*-methionine obtained from a tryptic digest of casein as described by du Vigneaud and Meyer (7) was acetylated as described above, but ethyl alcohol was used in the extraction of the acetylmethionine. After recrystallization from water the product melted at 111–111.5° (corrected) and had a specific rotation of  $[\alpha]_D^{25} = -16.1^\circ$ .

*Analysis*

3.126 mg. substance: 0.212 cc. N at 28.5° and 742 mm.

$\text{C}_7\text{H}_{13}\text{O}_2\text{NS}$ . Calculated, N 7.32; found, N 7.49

*Racemization of l-Methionine*

1 gm. of *l*-methionine was dissolved in 3.71 cc. of 2 N NaOH and diluted to 7 cc. with water. To this solution 2.2 cc. of acetic anhydride were then added in three equal portions with vigorous shaking. After the solution had stood at 37° for 5 hours, an amount of

6 N  $\text{H}_2\text{SO}_4$  equivalent to the NaOH used was added. After the solution was evaporated to dryness *in vacuo* the residue was extracted with hot ethyl acetate. 1.20 gm. of acetyl-*dl*-methionine were obtained. The product was identical with the acetyl derivative prepared from the synthetic *dl*-methionine described above.

#### Analysis

3.330 mg. substance: 0.219 cc. N at 27.5° and 747 mm.

$\text{C}_7\text{H}_{13}\text{O}_3\text{NS}$ . Calculated, N 7.32; found, N 7.34

#### Rate of Racemization of Acetyl-*d*-Glutamic Acid

Preliminary experiments indicated that glutamic acid was more resistant to racemization than either tryptophane or methionine. Furthermore, since we wished to determine the rate of racemization, it was felt advisable to prepare first the active acetyl-*d*-glutamic acid according to the method of Bergmann and Zervas (3) and study the racemization of the acetyl derivative rather than to start with the free amino acid as we did in the case of methionine. Because of the low specific rotation of acetyl-*d*-glutamic acid, it was necessary in studying the degree of racemization to hydrolyze the acetyl compound and to use the free glutamic acid for the rotation determination. For the latter the glutamic acid was dissolved in water containing 1 equivalent of hydrochloric acid.

To 20 gm. of acetyl-*d*-glutamic acid dissolved in 105.8 cc. of 2 N NaOH (2 mols), 140 cc. of acetic anhydride and enough water to make a total volume of 350 cc. were added. The flask was kept at 38° during the course of the experiment.

At various intervals 35 cc. portions were removed and each worked up in the following manner. In order to decompose the acetic anhydride and hence fix the length of time for the racemization reaction, 45 cc. of 6 N NaOH were added. 48.5 cc. of 6 N  $\text{H}_2\text{SO}_4$  were next added and the solution evaporated to dryness *in vacuo*. The acetylglutamic acid was extracted from the  $\text{Na}_2\text{SO}_4$  with hot absolute ethyl alcohol. After distillation of the alcohol, the compound was hydrolyzed by refluxing with 10 volumes of 2.5 N HCl for 1 hour. The glutamic acid hydrochloride was isolated by saturating the ice-cold solution with HCl gas. After crystallization seemed complete, an equal volume of absolute alcohol was added, the solution filtered, and the precipitate washed with a few cc. of alcohol and ether. A 90 per cent yield of the glutamic acid

hydrochloride was obtained. For the determination of the rotation the free glutamic acid was isolated and then dissolved in 1 equivalent of HCl. This procedure gave more consistent results than were obtained by determining the rotation of the hydrochloride as isolated. A control run with acetyl-*d*-glutamic acid in which no acetic anhydride was used showed no decrease in rotation whatsoever by this procedure. The results are shown in Chart I.

#### *Preparation of Formyl-dl-Phenylalanine*

Synthetic *dl*-phenylalanine was formylated by the method worked out by H. T. Clarke<sup>1</sup> for cystine. 28.3 gm. of *dl*-phenylalanine were dissolved in 300 cc. of 85 to 90 per cent formic acid and the solution warmed to 45°. 100 cc. of acetic anhydride were added gradually, the temperature rising to 70°. The solution was allowed to stand at this temperature for about 15 minutes and was then cooled in an ice bath and 200 cc. of water added. Crystallization of the formyl derivative began immediately. 20 gm. of formyl-*dl*-phenylalanine were obtained, which upon recrystallization gave 18 gm. of the pure compound melting at 168–169° (corrected). Fischer and Schoeller (8) report a melting point of 168.8–169.8°. By evaporation of the original mother liquor to a small volume and recrystallization of the material, 12.8 gm. more of the pure compound were obtained. The total amount of purified formyl-*dl*-phenylalanine was 91 per cent of the theoretical yield which is 10 per cent higher than that obtained by the formylation method of Fischer and Schoeller (8).

#### *Resolution of Formyl-dl-Phenylalanine*

The formyl-*dl*-phenylalanine was resolved by means of the brucine salt according to the directions given by Fischer and Schoeller (8). Both the formyl-*d*-phenylalanine and formyl-*l*-phenylalanine were obtained in excellent yields. Fischer reports that if the mother liquor from the brucine salt of the formyl-*d*-phenylalanine is kept free from moisture and placed in an ice box for a few days, the brucine salt of the levo compound will begin to crystallize out. We have obtained better yields by evaporating the mother liquor *in vacuo* to dryness and recrystallizing the residue from a minimum

<sup>1</sup> Personal communication.

amount of water. The brucine salt of the formyl-*d*-phenylalanine melted at 149–150° (corrected) and had a specific rotation of  $[\alpha]_D^{26} = -35.3^\circ$ , while the brucine salt of the levo isomer melted at 91–93° and had a specific rotation of  $[\alpha]_D^{25} = -7.3^\circ$ . Fischer did not report the rotation of the levo isomer.

The free formyl derivatives were freed from the brucine salts in the usual fashion and obtained in a 93 per cent yield. The formyl-*d*-phenylalanine melted at 167° and had a rotation of  $[\alpha]_D^{25} = -75.9^\circ$  in 95 per cent ethyl alcohol, which agree with the values given by Fischer and Schoeller (8).

#### *Preparation of d-Phenylalanine*

The free *d*-phenylalanine was prepared by hydrolysis of the formyl derivative by refluxing for 70 minutes with 12 volumes of 1 *N* HBr. The hydrobromide which was obtained by evaporation *in vacuo* to dryness was dissolved in absolute alcohol and  $\text{NH}_4\text{OH}$  added until the solution was neutral towards litmus. The phenylalanine crystallized out immediately. The crystallization was completed by cooling in an ice bath. A yield of 80 per cent of the theoretical was obtained. The *d*-phenylalanine had a rotation of  $[\alpha]_D^{26} = +33.5^\circ$  which is slightly lower than the maximum of  $+35.1^\circ$  reported by Fischer and Schoeller (8).

#### *Racemization of Sodium Salt of Formyl-L-Phenylalanine*

Preliminary experiments demonstrated that the formyl derivative was fairly resistant to racemization, and therefore a longer period of time for the reaction to take place was indicated. 1 gm. of the formyl-*L*-phenylalanine was dissolved in 2.6 cc. of 2 *N* NaOH. 3 cc. of water and 5 cc. of acetic anhydride were added and the solution kept at 37° for 24 hours. The formyl-*L*-phenylalanine recovered had a specific rotation of  $+17^\circ$ , showing that although considerable racemization had occurred, the reaction was much slower with the formyl derivative than with the acetyl derivative, the racemization of which will be shown later. 0.5 gm. of the above partially racemized product was treated again with the corresponding amounts of reagents and kept at 37° for 48 hours. The formylphenylalanine obtained after this treatment was completely inactive. By carrying out the reaction at 76° the racemization was complete within 12 hours.



Attempts to racemize the formyl-*l*-phenylalanine without using any sodium hydroxide were entirely negative. The formyl-phenylalanine recovered after standing with the acetic anhydride and water for 84 hours possessed practically the original rotation.

#### *Preparation of Acetyl-d-Phenylalanine*

4.5 gm. of *d*-phenylalanine were dissolved in 10.9 cc. of 2N NaOH. The solution was cooled in an ice bath and eight additions of 9 cc. of 2N NaOH and 0.9 cc. of acetic anhydride were made at intervals of 2 minutes, the mixture being continually shaken. After the solution had been allowed to stand at room temperature for some time, 27.64 cc. of 6N H<sub>2</sub>SO<sub>4</sub> were added and the solution evaporated *in vacuo* until the acetyl derivative began to crystallize out. 5.27 gm. of small rectangular plate-like crystals were obtained, the yield being 92 per cent of the theoretical. The product, after recrystallization from water, melted at 172° (corrected) and possessed a specific rotation of  $[\alpha]_D^{25} = -51^\circ$  in absolute alcohol. Knoop and Blanco (5) reported a melting point of 170° and a rotation of  $-51.8^\circ$  for *l*-acetylphenylalanine in absolute alcohol. They no doubt had the acetyl derivative of *d*-phenylalanine.

#### *Racemization of Acetyl-d-Phenylalanine*

The racemization of the acetyl-*d*-phenylalanine was carried out as in the case of the formyl derivative, corresponding amounts of sodium hydroxide and acetic anhydride being used. After the mixture had stood for 24 hours at 37°, an amount of H<sub>2</sub>SO<sub>4</sub> equivalent to the sodium hydroxide used was added, the mixture evaporated to dryness, and the acetylphenylalanine extracted with alcohol. The recovered product was completely inactive. Evidently the acetyl derivative is more readily racemized than the formyl. The inactive acetylphenylalanine melted at 150–151° and crystallized in needles. Knoop and Blanco (5) reported a melting point of 151°.

A run was made as above without the sodium hydroxide. The mixture was allowed to stand at 37° for 24 hours. At the end of that period the acetic anhydride was decomposed with excess NaOH, and H<sub>2</sub>SO<sub>4</sub> was added equivalent to the NaOH. The acetyl derivative which was then recovered as described above showed practically no change in rotation. Even after allowing

the acetic anhydride to act for 120 hours, the acetylphenylalanine showed a specific rotation of  $-48^\circ$  whereas that of the original material was  $-51^\circ$ .

Similar experiments were carried out with, in place of the sodium hydroxide, the equivalent amounts of the various substances to be tested. Experiments were carried out with sodium chloride, sodium acetate, sodium acetate plus sulfuric acid, and pyridine. The sodium chloride and sodium acetate plus sulfuric acid experiments led to only little racemization, whereas the sodium acetate and pyridine experiments gave complete racemization.

#### *Racemization of Monoacetyl-d-Arginine*

Monoacetyl-d-arginine was prepared according to the directions of Bergmann and Zervas (3). The crystalline product melted at  $269-270^\circ$  (corrected) and had a specific rotation of  $[\alpha]_D^{26} = +7.72^\circ$  in water.

1 gm. of the free acetyl-d-arginine was dissolved in water and 5 cc. of acetic anhydride were added. The mixture was kept at approximately  $37^\circ$  for 24 hours. At the end of 24 hours more water was added and the solution evaporated *in vacuo* to a small volume. This procedure was repeated three times and finally the material was taken up in 5 cc. of water from which the acetyl-arginine crystallized out in irregular broken platelets upon the addition of acetone as described by Bergmann and Zervas (3). The material was optically inactive and melted at  $108-110^\circ$  (corrected). The compound contains 2 molecules of water of crystallization.

#### *Analysis*

3.290 mg. substance: 0.661 cc. N at  $29^\circ$  and 748 mm.

$C_8H_{14}O_3N_4 \cdot 2H_2O$ . Calculated, N 22.22; found, N 22.34

#### *Preparation of N-Acetyl-l-Tyrosine*

15 gm. of l-tyrosine were dissolved in 42.5 cc. of 2 N NaOH and 25 cc. of water. After cooling the solution in ice, 200 cc. of 2 N NaOH and 20 cc. of redistilled acetic anhydride were added in eight equal portions with vigorous shaking and cooling between additions. After the mixture had stood at room temperature 40 minutes, 83.9 cc. of 6 N  $H_2SO_4$  were added. The mixture was allowed to remain in the refrigerator overnight, whereupon a small

amount of unreacted tyrosine precipitated out. The filtrate was evaporated to dryness *in vacuo*. The residue was then extracted with watery acetone and the extract evaporated to a thick syrup. Addition of water to the syrup caused it to crystallize in rod-like crystals. Crystallization from water gave 13.6 gm. of a product melting at 152–154° (corrected) which had a specific rotation for a 2 per cent aqueous solution of  $[\alpha]_D^{25} = 47.5^\circ$ . The compound gave a positive Millon reaction.

#### *Analysis*

3.296 mg. substance: 0.184 cc. N at 26° and 746 mm.

$C_{11}H_{13}O_4N$ . Calculated, N 6.27; found, N 6.25

#### *Preparation of Diacetyl-dl-Tyrosine*

15 gm. of *l*-tyrosine were dissolved in 166.6 cc. of 1 N NaOH and to this solution were added 60 cc. of redistilled acetic anhydride in 5 cc. portions. After the mixture was allowed to stand at 40° for 4 hours, 27.81 cc. of 6 N  $H_2SO_4$  were added, and the solution evaporated and extracted with acetone as above. The residue left after the evaporation of the acetone was crystallized from water in tufts of small needles. 7.8 gm. were obtained. The recrystallized material melted at 168–170° (corrected) and was optically inactive. In contrast to the monoacetyl derivative this compound gave only a slight suggestion of color with the Millon reagent after 10 minutes at room temperature and only a weak test after boiling.

#### *Analysis*

3.508 mg. substance: 0.170 cc. N at 30° and 746 mm.

$C_{13}H_{15}O_5N$ . Calculated, N 5.28; found, N 5.36

#### *Racemization of Diacetyl-l-Cystine*

1 gm. of diacetyl-*l*-cystine prepared according to the directions of Hollander and du Vigneaud (9) was dissolved in 6.6 cc. of 1 N NaOH and 3 cc. of redistilled acetic anhydride were added and the mixture kept at 35–40° for 3 hours. The solution diluted to a concentration of 0.2 per cent acetylcystine gave a specific rotation of  $[\alpha]_D^{30} = -10^\circ$  in contrast to that of the original diacetyl-*l*-cystine which was  $[\alpha]_D^{30} = -107.5^\circ$ . After standing a day, the racemized solution of acetylcystine began to deposit a precipitate which was identified as free sulfur, indicating that some decomposition had

taken place. We expect to investigate more closely this racemization and decomposition of cystine.

The rotation  $-10^\circ$  no doubt does not represent accurately the rotation of the remaining acetylcystine in the above experiment. However, attempts to isolate the acetylcystine and determine its rotation were unsatisfactory because of the amount of decomposition which had occurred. To show that racemization of acetylcystine really had occurred as well as decomposition, the racemization was carried out under even milder conditions, less acetic anhydride being used. The acetylcystine isolated from this reaction mixture was approximately 50 per cent racemized.

#### *Preparation of Acetyl-l-Proline*

10 gm. of *l*-proline isolated by Town's copper salt method (10) from gelatin was dissolved in 43.5 cc. of 2 N NaOH. To this solution kept cold in an ice bath, 98 cc. of 2 N NaOH and 9.8 cc. of acetic anhydride were added in seven equal portions. After the solution had stood for about an hour at room temperature, 47.2 cc. of 6 N  $\text{H}_2\text{SO}_4$  were added and the solution evaporated *in vacuo*. Water was added a number of times, each time followed by distillation to remove the acetic acid. It was finally reduced to dryness and the residue extracted with warm absolute alcohol. The alcohol was evaporated and the residue recrystallized from 15 cc. of water. After filtration, the crystalline product was dried for 2 days over  $\text{P}_2\text{O}_5$  in a vacuum desiccator. The weight of this product was 9.2 gm. It melted at  $116-117^\circ$  (corrected).

Upon recrystallization from water diamond-shaped prisms were obtained melting at  $81-82^\circ$  (corrected) after being air-dried. It was shown that this compound contained 1 molecule of water of crystallization and that upon drying to constant weight *in vacuo* at  $58^\circ$  the product melted at  $116-117^\circ$  (corrected). By recrystallizing the compound from absolute alcohol and ether, elongated triangular prisms melting at  $115-117^\circ$  (corrected) were obtained. The dry material had a specific rotation of  $[\alpha]_D^{27} = -106.7^\circ$ .

#### *Analysis*

3.217 mg. substance: 0.257 cc. N at  $27.5^\circ$  and 746 mm.

$\text{C}_7\text{H}_{11}\text{O}_2\text{N}$ . Calculated, N 8.91; found, N 8.90

*Attempted Racemization of Acetyl-L-Proline*

To 4.71 gm. of acetylproline 15 cc. of 2 N NaOH and 19 cc. of acetic anhydride were added. The solution was allowed to stand at 38° for 30 hours. At the end of this period, 5 cc. of 6 N H<sub>2</sub>SO<sub>4</sub> were added and the solution evaporated *in vacuo* to a small volume. 50 cc. of water were added and again distilled off. This was repeated twice more before bringing the material to dryness. The residue then was extracted with warm alcohol and the acetylproline isolated as before. The material had the same crystalline form and melting point as given for the acetyl-L-proline described above and was optically active. The specific rotation of the material was  $[\alpha]_D^{27} = -105^\circ$  which agrees with the specific rotation of the acetyl-L-proline already given.

## SUMMARY

The racemization reaction found by du Vigneaud and Sealock with the sodium salt of acetyltryptophane in aqueous solution with acetic anhydride has been extended to other amino acids.

It has been found that certain representative amino acids such as glutamic acid, phenylalanine, methionine, tyrosine, and arginine can be completely racemized by this reaction. It has furthermore been shown that the formyl derivative can also be racemized.

The conditions of the reaction have been studied and the rate of reaction of acetylglutamic acid has been determined.

It has also been found that during racemization of acetylcystine by this method decomposition occurs.

No racemization was obtained with proline.

The racemized acetyl derivative of naturally occurring methionine was shown to be identical with the acetyl derivative of the synthetic compound.

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## THE ACTION OF COPPER IN IRON METABOLISM\*

BY C. A. ELVEHJEM AND W. C. SHERMAN

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)

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The importance of copper as a supplement to iron for hemoglobin regeneration in anemic rats was first demonstrated 4 years ago by Hart, Steenbock, Waddell, and Elvehjem (1). This work showed definitely that in the presence of copper, soluble inorganic iron salts can be used directly for hemoglobin formation. Since that time a number of workers have studied factors affecting hemoglobin production in rats rendered anemic by whole milk diets. Most of this work has verified the original conclusions concerning the importance of copper, and today, although there are some who still feel that copper is not the only element in addition to iron which possesses hematopoietic properties, practically all workers agree that copper is an active agent in hemoglobin synthesis.

In most of these studies the activity of copper has been measured by comparing the increase in the hemoglobin content of the blood of anemic rats when pure iron alone is fed with the improvement obtained when the iron is accompanied by traces of copper. Aside from the fact that the hemoglobin molecule does not contain copper (2), nothing is known about the action of this element. In other words, we know that copper is necessary for the production of the finished blood pigment, but we have no understanding of how it functions in this interesting synthesis.

In this paper we wish to present the results obtained when special emphasis was placed on the action of copper on the storage and utilization of iron in the animal body. Since the liver and

\* A preliminary report of this work was presented before the Twenty-sixth meeting of the American Society of Biological Chemists at Philadelphia (*J. Biol. Chem.*, 97, p. xvi (1932)).

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spleen are known to be storage centers for iron a study has been made of the changes brought about in the iron content of these organs when copper was added to the diet of anemic animals and the iron thereby made available for hemoglobin formation.

#### EXPERIMENTAL

Rats were used throughout the entire investigation and were rendered anemic according to the technique described by Elvehjem and Kemmerer (3). The essential points in this method are: the young together with the mothers are placed on screens at birth, the mother is fed milk in the same cage but removed to another cage daily to receive the stock ration, and the young are weaned at 21 days of age when they are placed on whole cow's milk.

The young raised under these conditions become anemic much earlier than rats raised to weaning in an ordinary stock colony. In order to determine whether this difference is due to a reduced iron storage during the suckling period, the total iron content of a number of young was determined at short intervals between birth and weaning. For the purpose of comparison a similar study was made on a group of young raised in the ordinary way. A total of six litters was used; three were raised on screens and three on shavings. One rat in each litter was killed at birth, and one when the litter was 6, 14, 19, and 28 days old. The animals were dried and analyzed for iron.

Curves showing the variations in the total iron per rat and the mg. of Fe per gm. of dry material during the nursing period for the two groups are given in Chart I. The curve representing the iron content of rats reared on shavings and in the presence of the mother's ration is very similar to the curve given by Smythe and Miller (4) for normal young. It also substantiates their conclusions, that the absolute amount of iron in each rat increases with age, although the percentage of iron decreases during the same period.

The curves for the rats raised on shavings and on screens are quite similar until the animals are 14 days old. Even the rats raised on screens show an increase in total iron during the nursing period. When the rats are 10 days old the total iron content has increased to about twice the amount present at birth. The average increase for a number of rats during this time was found

to be 0.25 mg. of Fe. Since there is no other source of iron, this amount must come from the mother's milk. This indicates that the young rats must consume more than 100 cc. of milk in 10 days or that the iron content of rat milk is considerably higher than that of cow's milk. An attempt was made to determine the iron content of rat milk by removing the milk from the stomachs of

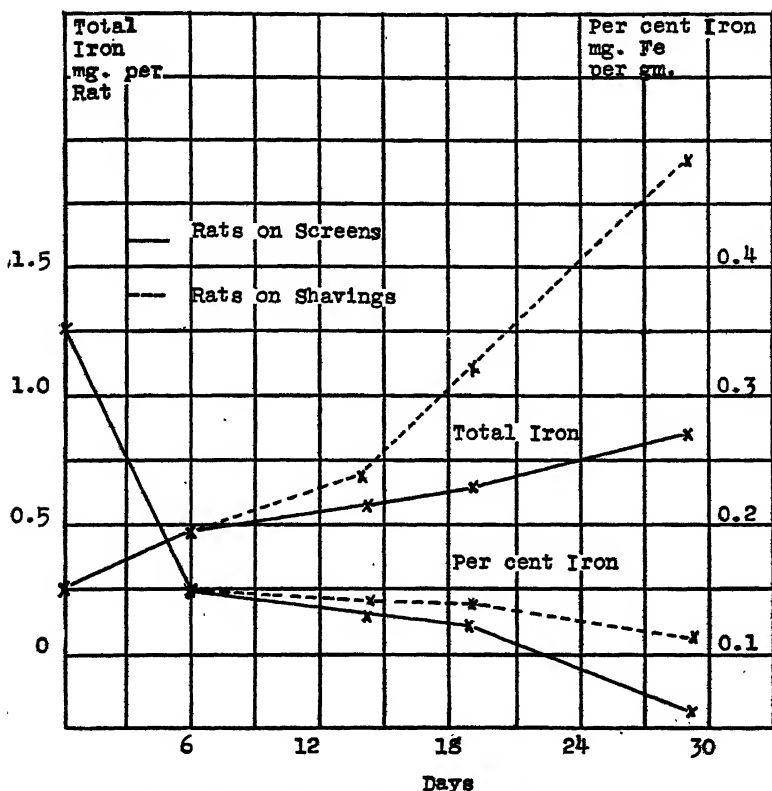


CHART I. The effect of postnatal care on the iron content of young rats.

day old rats directly after nursing. This method cannot be considered very accurate, but the individual results were found to be fairly constant; namely, 50 mg. of Fe per kilo of dry milk. This is about 3 times that of cow's milk.

The latter part of the curve for the rats reared on screens readily explains the early development of anemia in these animals.



The rats raised under the restricted condition store much less iron than the others and are unable therefore to maintain the hemoglobin content of the blood for as long a period after weaning. If the rats are weaned at 21 days those raised by the old method contain almost twice as much iron as the others. Some workers do not wean their rats until they are 28 days old; in this case the difference would be still greater. The curves for the percentage of iron do not differ to such a large extent because the rats raised on screens become anemic much earlier and therefore do not grow as rapidly. The decreased weight causes an increase in the percentage of iron.

The difference which has been demonstrated for the total iron content of the entire rat also holds in the case of the liver. The livers from rats raised in contact with the mother's ration, weaned at 21 days, and kept on milk alone for 2 weeks contained 0.25 mg. of Fe, while the livers from rats raised on screens and treated in the same manner contained only 0.10 mg. of Fe. The rats used in the following investigation were therefore well suited for our studies, because the store of iron present in the livers at birth and available for hemoglobin formation was entirely depleted before additional iron was added to the diet.

The following procedure was used for studying the action of copper. Litters of six rats each were weaned and placed on whole cow's milk. When the hemoglobin had decreased to 2 to 3 gm. per 100 cc. of blood, usually 14 days after weaning, two rats in the litter were killed by decapitation and the liver and spleen removed carefully. The remaining four rats were given 0.5 mg. of Fe as purified  $\text{FeCl}_3$  daily. After 2 weeks of iron feeding two of these rats were killed and the livers and spleens removed. The iron was taken away from the two remaining rats and they were fed 0.05 mg. of Cu as  $\text{CuSO}_4$ , daily. After 2 weeks these rats were killed for the removal of the liver and spleen. The organs were dried, weighed, and analyzed for iron. The hemoglobin content of the blood of the rats was determined weekly. A total of five litters was used for this study. The results are given in Table I.

When pure iron is fed to anemic rats for a period of 2 weeks there is no increase in the amount of hemoglobin in the blood, but there is a decided increase in the total iron content of the liver and spleen. The total iron in the liver increased from an average

of 0.12 to 0.46 mg. of Fe and that of the spleen from 0.024 to 0.068 mg. of Fe. The increase when calculated as percentage of iron is not quite so large because the average weight of the liver and spleen increased during the 2 week period.

When the iron was taken away and 0.05 mg. of copper substituted, the average increase in hemoglobin for the 2 weeks was 2 gm. as compared with 0.1 gm. when the iron was fed. Most of the increase in the hemoglobin took place during the 1st week of copper feeding, and in many cases the hemoglobin regeneration had reached a maximum at 1 week and decreased thereafter, due to lack of sufficient iron. After 2 weeks of copper feeding the iron content of the liver had dropped to approximately the same level

TABLE I

*Effect of Copper on Iron Content of Livers and Spleens from Rats*

Each figure is the average of ten results.

	Change in Hb	Liver			Spleen		
		Weight	Fe	Fe per gm. dry liver	Weight	Fe	Fe per gm. dry spleen
	gm.	gm.	mg.	mg.	gm.	mg.	mg.
Anemic rats . . . . .		0.694	0.126	0.184	0.034	0.024	0.775
Rats fed 0.5 mg. Fe for 14 days . . . . .	+0.10	1.030	0.464	0.465	0.055	0.068	1.470
Rats fed 0.5 mg. Fe for 14 days then 0.05 mg. Cu alone for 14 days . . . . .	+2.00	0.970	0.119	0.124	0.112	0.058	0.509

as was present before any iron was fed. The total iron content of the spleen decreased but very little. The percentage of iron decreased due to a decided increase in the weight of the spleen. A pronounced increase in the size of the spleen was noted in all rats when they were changed from a diet of milk and iron to one of milk, iron, and copper. This increase in the size of the spleen with copper feeding is very interesting and further study is needed to elucidate the significance of the change.

From these results it is evident that in the absence of copper inorganic iron is readily assimilated and stored in the liver and spleen. The iron so stored cannot be used for hemoglobin formation until copper is supplied, when the greater part of the iron in

the liver is removed and built into hemoglobin of the blood. Copper is not necessary for iron assimilation but it is necessary for the synthesis of hemoglobin.

In the work which has just been described iron was fed for 2 weeks, then discontinued and followed by copper feeding immediately. A few experiments were conducted, therefore, to show what happens when the rats are continued on milk alone after the iron feeding. The results obtained with six typical animals are given in Table II. All the rats received 0.5 mg. of Fe for 2 weeks.

TABLE II

*Iron Content of Livers of Rats Continued on Milk Alone and Milk Plus Copper after Iron Feeding*

Rat No.	Treatment	Hemoglobin		Total Fe in liver	Fe per gm. dry liver
		14 days	21 days		
		gm.	gm.	mg.	mg.
400	0.5 mg. Fe daily for 14 days	2.98		0.630	0.484
612		2.87		0.312	0.441
401	0.5 mg. Fe daily for 14 days followed by milk alone for 7 days	3.47	2.10	0.270	0.279
613		3.85	3.87	0.303	0.291
402	0.5 mg. Fe daily for 14 days followed by milk + 0.05 mg. Cu daily for 7 days	2.70	5.91	0.121	0.112
611		2.13	6.66	0.113	0.131

Two of the animals were killed, two were continued on milk alone for 1 week, and two received 0.05 mg. of copper for 1 week before the organs were removed. It is readily seen that although there is some loss of iron in the liver of rats continued on milk alone, it is not nearly as great as when copper is fed. The loss observed in the milk-fed animals is undoubtedly due to a slow filtration of the iron into the channels of excretion, because there is no increase in the hemoglobin of the blood. The increase in the hemoglobin of the blood of the animals fed copper for 1 week is higher than the average given in Table I but as has already been stated, the figure is generally higher at the end of the 1st week than after 2 weeks of copper feeding.

Since inorganic iron was used in these experiments, the question of the possibility of rats assimilating and storing organic forms of iron naturally arises. Elvehjem (5) has shown recently that organic iron, in the absence of copper, is equally as ineffective as inorganic iron for hemoglobin formation, while in the presence of copper the regeneration with organic iron is much inferior to that with inorganic iron. The analysis of the livers from a few animals led to the conclusion that the decreased activity of the organic iron is due to the inability of the animal to assimilate iron in the form of hematin. This conclusion is amply substantiated by a few typical results given in Table III. Two of the animals received 0.5 mg. of Fe per day as hematin and two the same amount of iron as  $\text{FeCl}_3$  for 7 days. The livers from those receiving ferric

TABLE III  
*Effect of Form of Iron on Its Storage in the Liver*

Litter 440, weaned when 21 days old and started when 36 days old.

Rat No.	Fe addition for 7 days	Weight of liver	Total Fe content	Fe per gm. of liver
	mg.	gm.	mg.	mg.
440	0.5 as hematin	0.758	0.064	0.085
441	0.5 " "	0.723	0.077	0.106
442	0.5 as $\text{FeCl}_3$	0.520	0.210	0.500
443	0.5 " "	0.744	0.364	0.488

chloride contained 5 times as much iron as those from rats given hematin. This method may be used for testing the availability of any iron compound which can be prepared free from copper.

A second experiment was conducted in order to determine how much iron must be fed to allow hemoglobin regeneration and iron storage to take place at the same time. Graded levels of inorganic iron were fed with and without copper to anemic rats for 14 days, after which the rats were killed and the livers removed for iron analysis. The hemoglobin was determined weekly. The results are given in Chart II.

When the iron was fed alone the hemoglobin of the blood remained unchanged but the amount of iron stored in the liver was proportional to the amount of iron fed. When the iron was fed with copper the rate of hemoglobin formation was dependent upon

the iron intake, but the iron in the liver did not increase until 0.3 mg. of Fe was fed daily. In other words, when copper is present the inorganic iron is built directly into hemoglobin, and the amount in excess of what is needed for hemoglobin production is stored in the liver until the optimum level of iron storage in this organ is reached. It is impossible to increase the iron content of

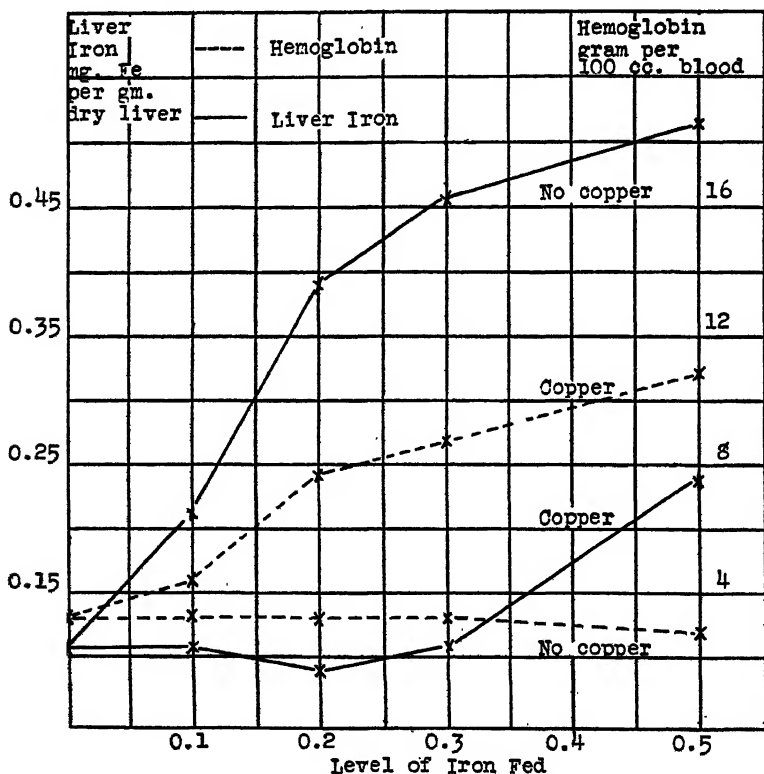


CHART II. The effect of copper on the storage of iron in the liver and the hemoglobin content of the blood when graded levels of iron are fed.

rat livers much above 1 mg. per gm. of dry matter by prolonged iron feeding when fed with or without copper.

Copper must therefore be active in the synthesis of hemoglobin from inorganic iron or in some process which affects this transformation.

## DISCUSSION

It is interesting to compare the results presented in this paper with those published by Williamson and coworkers (6-8) a few years ago. They used animals which were not depleted in their original iron reserves and did their work before the importance of copper was known; nevertheless, the conclusions made from the two studies are comparable in many ways.

Williamson and Ets (6) demonstrated that inorganic iron, whether given by mouth, subcutaneously, or intravenously, increased the store of iron in the liver and spleen, but had no effect upon the rate of hemoglobin regeneration. They (7) also showed that rats fed ferric citrate in addition to a standard diet for some time did not recover any faster from anemia induced by bleeding than the controls not given any iron. The stored iron could not be used for hemoglobin formation. Williamson and Ewing (8) concluded, "The fault lies not with the absorption of iron, as was supposed by Bunge and his followers, but with its ability to be converted into hemoglobin."

The results presented in this paper show that the iron content of the livers of rats can be reduced to 0.1 mg. of Fe per gm. of dry matter by feeding a milk diet and increased without altering the hemoglobin of the blood to about 1 mg. of Fe by feeding inorganic iron. The iron so stored is readily converted to hemoglobin when copper is fed. Williamson's animals probably lacked sufficient copper to bring about this change. When he and Ewing (8) fed liver, a reserve of iron was built up which could be used for hemoglobin regeneration. In this case the iron was available because the liver also supplied copper.

A few workers have studied the assimilation and utilization of iron after the importance of copper was discovered. Cook and Spilles (9) showed that the addition of copper to an iron-low diet resulted in a depletion of the reserve iron in the spleen. When copper was added to a dietary supplied with sufficient iron, the amount of splenic iron was greatly increased, but iron alone did not bring about this augmentation. The results in this paper show that the iron content of the spleen is increased with iron feeding when the original reserve of iron is first depleted.

Cunningham (10) studied the effect of copper feeding on the iron content of the livers and the bodies of rats. His results are

far from conclusive, but he suggests that the inclusion of copper in the diet has the effect of lowering the iron content of the liver and increasing that of the body.

In a more recent paper Josephs (11) concludes that copper has no influence on the retention of iron but does influence the proportion of the retained iron found in the hemoglobin. He presents figures to show that when rats are receiving a plentiful supply of iron 80 per cent of the retained iron goes into hemoglobin formation and that this amount is increased to 100 per cent under the influence of copper. This clearly indicates that his rats were not depleted in their copper stores. In the absence of copper none of the retained iron can be built into blood hemoglobin. He concludes that copper decreases the non-hemoglobin iron of the tissues (total body iron minus blood hemoglobin iron). This is not strictly true because the tissues contain hemoglobin as well as the blood. He later suggests that copper influences mainly the mobile portion of the non-hemoglobin iron and that the majority of this iron is found in the liver. This conclusion is in agreement with our results. He emphasizes the fact that the fixed iron in the liver does not fall below a certain concentration which is maintained at all cost. We have also found that the iron content of the liver rarely falls below 0.1 mg. per gm. of dry tissue. When this level is reached the animal fails to grow.

All these workers reached the same general conclusion, namely, that copper mobilizes the iron in the liver, but the results in this paper give a definite account of the retention and utilization of inorganic iron in the absence and presence of copper.

#### SUMMARY AND CONCLUSIONS

1. Chart I is presented to show the effect of postnatal care on the iron content of young rats.

2. The addition of pure iron to the milk diet of anemic rats, which had been well depleted in their reserve of iron, had no effect on the hemoglobin content of the blood, but increased the total iron content of the liver and spleen to a large extent. When the iron was replaced by copper, the store of iron in the liver was used directly for building blood hemoglobin. The copper caused only a slight decrease in the iron content of the spleen but produced a definite increase in the size of this organ.

3. Inorganic iron ( $\text{FeCl}_3$ ) was found to be much more readily assimilated and stored in the liver than organic iron (hematin).

4. When graded levels of inorganic iron were fed in the absence of copper, the hemoglobin content of the blood remained unchanged and the amount of iron stored in the liver was proportional to the amount of iron fed. In the presence of copper, the rate of hemoglobin formation was dependent upon the iron intake and the liver showed no iron storage until 0.3 mg. or more of iron was fed.

5. Copper does not affect the assimilation of iron but does function in the conversion of inorganic iron into hemoglobin.

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## STUDIES ON RACEMIZATION

### XI. ACTION OF ALKALI ON POLYPEPTIDES

BY P. A. LEVENE AND ROBERT E. STEIGER

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

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In a previous publication<sup>1</sup> a report was made on the behavior towards alkali of di- and tripeptides entirely composed of optically active amino acids. It had been established that the dipeptide dextro-leucyl-dextro-leucine, when submitted to the action of 10 equivalents of 1.0 N sodium hydroxide at 25° over a prolonged period of time (8 days), did not suffer any racemization, whereas the tripeptides levo-alanyl-dextro-leucyl-dextro-leucine and dextro-leucyl-dextro-leucyl-dextro-leucine, when subjected to the same treatment, were partly racemized. The conclusion was drawn that migration, under the influence of alkali, of the hydrogen atoms attached to asymmetric carbon atoms takes place only on the central amino acids of a polypeptide chain, the terminal amino acids which carry the free amino or the carboxyl group remaining wholly unaltered. The prediction of Dakin<sup>2</sup> that racemization in polypeptides would take place only in systems composed of at least three amino acids was thus substantiated.

No loss of optical activity having been observed when the two tripeptides mentioned above were treated with alkali of lower concentration (2 equivalents of 0.2 N sodium hydroxide), an additional conclusion was reached; namely, that a minimum concentration of alkali was required to produce the significant change to some appreciable degree. Observations in this direction had already been made by Levene and Pfaltz<sup>3</sup> with polypeptides

<sup>1</sup> Levene, P. A., Steiger, R. E., and Marker, R. E., *J. Biol. Chem.*, **93**, 605 (1931).

<sup>2</sup> Dakin, H. D., *J. Biol. Chem.*, **13**, 362 (1912-13).

<sup>3</sup> Levene, P. A., and Pfaltz, M. H., *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926).

composed of levo-alanine and glycine. They had observed that the racemization increased with the concentration of the sodium hydroxide solutions used. These, however, (and sometimes the temperatures) were kept low because of the simultaneous, quite rapid hydrolysis of the peptides under the influence of the alkali. The leucine tripeptides studied later were highly resistant to the action of even 1.0 *N* sodium hydroxide; in fact, they were selected for study for precisely that reason. With little or no hydrolysis to the optically stable dipeptide taking place, it seemed that these compounds—or better, their central amino acid unit—should be racemized in alkali of such high concentration to a much greater extent than was actually the case. The question naturally arises whether the racemization of any central amino acid unit of a polypeptide chain varies greatly with the nature of the alkyl (or aryl) group attached to the asymmetric carbon atom under consideration. An answer to this may be found in a forthcoming publication.

The present work was undertaken in order to see to what extent the degree of racemization of such an amino acid (*e.g.*, dextro-leucine) is dependent on the nature of the adjacent or all other amino acid units of the molecule or varies with the position occupied by this amino acid within the chain. Two tetrapeptides (of fair resistance to the hydrolytic action of 1.0 *N* sodium hydroxide), just obtained, have now been examined with regard to their optical stability under the conditions specified.

The results are given in Table I. The preparation of the tetrapeptides and of the intermediates is described in the experimental part. The data of the racemization and the control hydrolysis experiments are recorded in Tables II and III.

It can be seen from Table I that there is considerable loss of optical activity when the leucyl unit of the tripeptide dextro-leucyl-dextro-leucyl-dextro-leucine, carrying the carboxyl group, is replaced by glycyl-glycine. For the tetrapeptide glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine, which contains two leucine units susceptible to racemization, the loss of optical activity was higher than that observed either for the parent substance or for the preceding compound. It must be borne in mind, however, that the figures given in Table I do not represent the racemization values of the central leucine units of the peptides, since in the

calculations no allowance has been made for the terminal asymmetric amino acids which, as has been shown, retain their optical activity.

## EXPERIMENTAL

## 1. Preparation of Compounds

*Levo- $\alpha$ -Bromoisocaproeryl-Glycyl-Glycine*

46 gm. (0.4 mol) of finely powdered glycine anhydride<sup>4</sup> and 400 cc. of 1.0 N sodium hydroxide (0.4 mol) were placed in a well stoppered bottle and the mixture was shaken at room temperature

TABLE I  
*Action on Peptides of 1.0 N Sodium Hydroxide at 25°*

Compound	Loss of optical activity after 8 days with 10 mols 1.0 N NaOH at 25°
	<i>per cent</i>
<i>d</i> -Leucyl- <i>d</i> -leucine .....	0*
<i>l</i> -Alanyl- <i>d</i> -leucyl- <i>d</i> -leucine .....	5*
<i>d</i> -Leucyl- <i>d</i> -leucyl- <i>d</i> -leucine .....	4*
<i>d</i> -Leucyl- <i>d</i> -leucyl-glycyl-glycine .....	19
Glycyl- <i>d</i> -leucyl- <i>d</i> -leucyl- <i>d</i> -leucine .....	23

\* Figures previously reported.

until a clear solution was obtained (hydrolysis to the dipeptide). This was then cooled in an ice and water mixture during treatment with 86 gm. (0.4 mol) of levo- $\alpha$ -bromoisocaproeryl chloride and 400 cc. of 1.0 N sodium hydroxide (0.4 mol), these reagents being added in equivalent proportions with continuous shaking. 104 cc. of 5.0 N hydrochloric acid (0.52 mol) were then added and a crystalline precipitate of the bromo compound was obtained at once. This was filtered off, washed with ice water, then with petroleic ether, and was dried in a vacuum desiccator over phosphorus pentoxide. Yield, 99.0 gm. (80 per cent of the theory). This material was dissolved in 693 gm. of absolute ethyl acetate (7 parts by weight) at boiling temperature. Norit was added and the

<sup>4</sup> Prepared from glycine ethyl ester hydrochloride in the way indicated by Fischer, E., *Ber. chem. Ges.*, 39, 2930 (1906).

solution was filtered while hot. Crystallization set in immediately. The crystals were filtered off at room temperature and were well washed with ethyl acetate. They were dried in a vacuum oven at 50° until free of solvent. They weighed 87.0 gm. For analysis and rotation a fraction of this material was recrystallized twice from boiling absolute ethyl acetate. It then sintered and melted between 130–132° (uncorrected).

No. 493.	4.545 mg. substance:	6.540 mg. CO <sub>2</sub> and 2.255 mg. H <sub>2</sub> O
	5.455 " "	: 0.445 cc. N (24°, 756.7 mm.)
	6.000 " "	: 3.625 mg. AgBr (Carius)
C <sub>10</sub> H <sub>17</sub> O <sub>4</sub> N <sub>2</sub> Br (309.08).	Calculated.	C 38.83, H 5.55, N 9.06, Br 25.86
	Found.	" 39.23, " 5.55, " 9.34, " 25.70

It had the following rotations.

$$[\alpha]_D^{20} = \frac{-5.93^\circ \times 100}{4 \times 3.0824} = -48.1^\circ \text{ (in absolute alcohol)}$$

0.4636 gm. in 15.04 cc. of solution (0.1 M)

$$[\alpha]_D^{20} = \frac{-2.25^\circ \times 100}{4 \times 1.5453} = -36.4^\circ \text{ (in water)}$$

0.2318 gm. in 15.00 cc. of solution (0.05 M)

Abderhalden and Fodor<sup>5</sup> found for dextro- $\alpha$ -bromoisocapro-nyl-glycyl-glycine  $\alpha_D^{20} = +0.83^\circ$  (0.5 dm. tube) or  $[\alpha]_D^{20} = +47.01^\circ$ , employing a solution of 0.2789 gm. in absolute alcohol, the weight of the solution being 6.3659 gm. They found  $\alpha_D^{20} = +0.23^\circ$  (0.5 dm. tube) or  $[\alpha]_D^{20} = +31.17^\circ$ , employing a solution of 0.2250 gm. in water, the weight of the solution being 15.4591 gm. Their material melted at 130–132°.

#### *Dextro-Leucyl-Glycyl-Glycine*

A solution of 81 gm. of levo- $\alpha$ -bromoisocapro-nyl-glycyl-glycine (crystallized once from ethyl acetate) in 810 gm. of ammonium hydroxide of sp. gr. 0.90 was kept at room temperature for 3 days. It was then diluted with water and concentrated to a small volume under reduced pressure. Water was then repeatedly added and evaporated off each time in order to remove the ammonia. The

<sup>5</sup> Abderhalden, E., and Fodor, A., *Ber. chem. Ges.*, 49, 564 (1916).

syrupe residue was diluted with some water and the solution obtained was clarified with norit. A large volume of absolute alcohol was added to the filtrate. Crystallization of the tripeptide set in after prolonged stirring of the solution. The mixture was left to stand for 2 days in the ice box. The crystals were filtered off and thoroughly washed with hot absolute alcohol. They were dried in a vacuum desiccator over phosphorus pentoxide. Yield, 55 gm. (85.6 per cent of the theory). This material contained traces of chlorine and ammonia. For analysis and rotation it was recrystallized twice from water and alcohol. In each case the substance was dissolved in a small amount of water. The solution was clarified with norit and about 9 volumes of absolute alcohol were added to the filtrate at room temperature. After crystallization had started, the mixture was cooled for 24 hours in ice and water. The crystals were thoroughly washed with hot absolute alcohol; they were then dried in a vacuum oven at 60° and finally in a vacuum desiccator over phosphorus pentoxide. For analysis they were redried at 100°.

No. 496.	5.116 gm. substance:	9.190 gm. CO <sub>2</sub> and 3.570 gm. H <sub>2</sub> O
	0.1000 gm. " :	12.21 cc. 0.1 N HCl (Kjeldahl)
	21.700 gm. " :	2.33 cc. N (28.6°, 754 mm.) (Van Slyke)
C <sub>10</sub> H <sub>19</sub> O <sub>4</sub> N <sub>3</sub> (245.17).	Calculated.	C 48.95, H 7.81, N 17.14, NH <sub>2</sub> 5.71
	Found.	" 48.98, " 7.80, " 17.11, " 5.79
		Moisture, none

It had the following rotation.

$$[\alpha]_D^{20} = \frac{-9.69^\circ \times 100}{4 \times 4.9033} = -49.4^\circ \text{ (in water)}$$

0.7355 gm., dry, in 15.00 cc. of solution (0.2 M)

Abderhalden and Fodor<sup>6</sup> found for a levo-leucyl-glycyl-glycine, twice recrystallized from water and alcohol,  $[\alpha]_D^{20} = +43.42^\circ$  in water, and for a fraction of tripeptide isolated from the second mother liquor of crystallization  $[\alpha]_D^{20} = +45.90^\circ$  in water. A sample from another of their operations showed a rotation of  $[\alpha]_D^{20} = +44.79^\circ$  in water.

<sup>6</sup> Abderhalden, E., and Fodor, A., *Ber. chem. Ges.*, 49, 565 (1916).

*Levo- $\alpha$ -Bromoisocaprolyl-Dextro-Leucyl-Glycyl-Glycine*

A solution of 88.3 gm. (0.36 mol) of dry dextro-leucyl-glycyl-glycine in 360 cc. of 1.0 N sodium hydroxide (0.36 mol) was cooled in an ice and water mixture during treatment with 77 gm. (0.36 mol) of levo- $\alpha$ -bromoisocaprolyl chloride and 360 cc. of 1.0 N sodium hydroxide (0.36 mol), these reagents being added in equivalent proportions with continuous shaking. 10 cc. of ether and then 100 cc. of 5.0 N hydrochloric acid (0.5 mol) were added to the solution. The bromo compound precipitated as a pasty mass. The mixture was then shaken at room temperature until crystallization took place. The material was filtered off and washed with ice water and with petroleic ether. It was then dissolved in absolute alcohol and reprecipitated with a large amount of water. The material obtained (no yield is given, an undetermined amount having been accidentally lost) melted at 186–187° (uncorrected) with decomposition. Its rotation was  $[\alpha]_D^{25} = -4.97^\circ$  (in absolute alcohol). On recrystallization from a mixture of absolute ethyl acetate and alcohol (the procedure given below was followed in its essentials) a material of higher rotation, yet still melting at 186–187° (uncorrected), was obtained.

$$[\alpha]_D^{25} = \frac{-0.96^\circ \times 100}{4 \times 4.2220} = -5.68^\circ \text{ (in absolute alcohol)}$$

0.6333 gm. in 15.00 cc. of solution (0.1 M)

This material was recrystallized once more from the same mixture of solvents. There was no increase in rotation. It melted at 188° (uncorrected) or at 190° (corrected), with alteration. The fused substance then decomposed rapidly.

No. 494.	5.405 mg. substance:	9.028 mg. CO <sub>2</sub> and 3.100 mg. H <sub>2</sub> O
	0.1000 gm. " :	7.16 cc. 0.1 N HCl (Kjeldahl)
	0.0969 " " :	0.0433 gm. AgBr (Carius)
C <sub>18</sub> H <sub>31</sub> O <sub>5</sub> N <sub>2</sub> Br (422.16). Calculated. C 45.48, H 6.69, N 9.95, Br 18.93		
	Found. " 45.54, " 6.41, " 10.02, " 19.01	

The substance used for amination was highly purified. It was obtained from 73.5 gm. of original material of rotation  $[\alpha]_D^{25} = -4.97^\circ$ , which was recrystallized four times in the following way:

Absolute alcohol was added at boiling temperature to suspensions of 1 part of bromo compound in 2 parts by weight of absolute ethyl acetate until the materials were completely dissolved. When necessary, norit was added and the solutions were filtered while hot. To these, 5.3 parts by weight (in respect to the bromo compound) of absolute ethyl acetate were then added. For the first and second crystallizations the solutions were simply cooled to room temperature, but subsequently the mixtures were cooled in ice and water. The crystals were washed with an ethyl acetate-alcohol mixture (1:1, by volume) and finally with heptane. They were dried in a vacuum oven at 60° and then in a vacuum desiccator over phosphorus pentoxide and sodium hydroxide. The yields were as follows: 73.5 gm. → 56.0 gm. → 32.1 gm.; 31.0 gm. → 24.6 gm. → 19.7 gm. The rotations of the materials obtained after the second and fourth recrystallizations were identical; namely,  $[\alpha]_D^{30} = -5.64^\circ$ , in absolute alcohol ( $\alpha_D^{30} = -0.95^\circ$ , 4 dm. tube; 0.6333 gm. in 15.03 cc. of solution).

*Dextro-Leucyl-Dextro-Leucyl-Glycyl-Glycine*

A solution of 18.7 gm. of pure levo- $\alpha$ -bromoisocaproonyl-dextro-leucyl-glycyl-glycine in 187 gm. of ammonium hydroxide of sp. gr. 0.90 was kept at room temperature for 4½ days. It was then diluted with water, treated with norit, and concentrated to dryness under reduced pressure. The material was suspended in a small amount of water and absolute alcohol was added to the volume of 1500 cc. This mixture was cooled for 12 hours in the ice box. The solid was filtered off, thoroughly washed with hot absolute alcohol, and while still moist placed in a vacuum desiccator over phosphorus pentoxide and kept there until completely dry. Yield, 11.5 gm. (72 per cent of the theory). This material was again purified in the way indicated above, with the cooling below room temperature omitted. It was then dried in a vacuum oven at 60° and finally over phosphorus pentoxide.

No. 497. 4.965 mg. substance: 9.710 mg. CO<sub>2</sub> and 3.700 mg. H<sub>2</sub>O  
 5.374 " " : 0.755 cc. N (31°, 753.7 mm.)

C<sub>18</sub>H<sub>30</sub>O<sub>5</sub>N<sub>4</sub> (358.27). Calculated. C 53.59, H 8.44, N 15.64  
 Found. " 53.33, " 8.34, " 15.65  
 Moisture, none



It had the following rotation.

$$[\alpha]_D^{20} = \frac{-1.14^\circ \times 100}{4 \times 3.568} = -8.0^\circ \pm 0.2^\circ \text{ (in pyridine-water)}$$

0.5374 gm. + 5.50 cc. of pyridine + water to volume 15.06 cc. (0.1 M solution)

*Chloroacetyl-Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine*

A solution of 8.4 gm. (0.0235 mol) of dry dextro-leucyl-dextro-leucyl-dextro-leucine (9.1 gm. of tripeptide containing 7.74 per cent of moisture)<sup>7</sup> in 23.5 cc. of 1.0 N sodium hydroxide (0.0235 mol) was cooled in an ice and water mixture during treatment with 8.0 gm. (0.0705 mol) of chloroacetyl chloride and 77.0 cc. of 1.0 N sodium hydroxide (0.0770 mol), these reagents being added in equivalent proportions with continuous shaking. 20 cc. of 5.0 N hydrochloric acid (0.1000 mol) were added and a crystalline precipitate of the chloro compound was obtained at once. The crystals were filtered off and washed with ice water and then with petroleic ether; they were again washed with water and with petroleic ether. This material was dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It weighed 10.2 gm. (theory, 10.2 gm.). The solution of this material in hot absolute alcohol was clarified with norit and filtered. Water was gradually added to the solution until crystallization set in. The mixture was left to cool slowly to room temperature and then a further amount of water was added. The crystals deposited were filtered off, washed with dilute alcohol, and then with petroleic ether. 8.0 gm. of dry substance were recovered. It melted at 198–199° (uncorrected), with no apparent alteration. After some time, however, the fused material suffered decomposition.

No. 492. 4.995 mg. substance: 10.100 mg. CO<sub>2</sub> and 3.610 mg. H<sub>2</sub>O

5.800 " " : 0.484 cc. N (24°, 758.8 mm.)

15.430 " " : 5.300 mg. AgCl (Carius)

C<sub>19</sub>H<sub>33</sub>O<sub>5</sub>N<sub>3</sub>Cl (433.78). Calculated. C 55.33, H 8.37, N 9.69, Cl 8.17

Found. " 55.14, " 8.08, " 9.58, " 8.49

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<sup>7</sup> Levene, P. A., Steiger, R. E., and Marker, R. E., *J. Biol. Chem.*, **93**, 615 (1931).

It had the following rotation.

$$[\alpha]_D^{20} = \frac{+2.98^\circ \times 100}{4 \times 1.0465} = +71.2^\circ \text{ (in absolute alcohol)}$$

0.1575 gm. in 15.05 cc. of solution

Abderhalden and Fleischmann<sup>8</sup> found for chloroacetyl-levo-leucyl-levo-leucyl-levo-leucine  $\alpha_D^{20} = -0.8^\circ$  (1 dm. tube) or  $[\alpha]_D^{20} = -76.19^\circ$ , employing a solution of 0.105 gm. in alcohol, the volume of the solution being 10 cc. Their material melted at  $193^\circ$  with decomposition.

*Glycyl-Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine*

A mixture of 7.0 gm. of purified chloroacetyl-dextro-leucyl-dextro-leucyl-dextro-leucine with 10 times this weight of ammonium hydroxide of sp. gr. 0.90 was kept at room temperature for 3 days (temperature about  $25^\circ$ ) during which time it was occasionally shaken. A cloudy solution was finally obtained; it was diluted with water and clarified with norit. The filtrate was concentrated under reduced pressure. The ammonia thus being removed, the tetrapeptide separated in the form of a voluminous gel. This was filtered off and thoroughly washed with absolute alcohol. The filtrate was concentrated under reduced pressure. It then yielded a second fraction of polypeptide. The two fractions were combined. This material weighed 4.7 gm. It was suspended in absolute alcohol and small amounts of ammonium hydroxide, of sp. gr. 0.90, were added gradually until an almost clear solution was obtained. This was clarified with norit. The filtrate was concentrated under reduced pressure for the removal of the ammonia and the recovery of the tetrapeptide. Alcohol was freely run into the suspension obtained while the concentration was continued. The substance was filtered off, thoroughly washed with absolute alcohol, and was then dried in a vacuum desiccator over phosphorus pentoxide.

No. 495. 5.251 mg. substance: 11.075 mg.  $\text{CO}_2$  and 4.270 mg.  $\text{H}_2\text{O}$   
4.030 " " : 0.477 cc. N ( $29^\circ$ , 755.4 mm.)

$\text{C}_{20}\text{H}_{38}\text{O}_5\text{N}_4$  (414.33). Calculated. C 57.92, H 9.24, N 13.52  
Found. " 57.51, " 9.09, " 13.29  
Moisture, 0.73 per cent

<sup>8</sup> Abderhalden, E., and Fleischmann, R., *Fermentforschung*, 9, 529 (1926-28).

It had the following rotation.

$$[\alpha]_D^{20} = \frac{+6.7^\circ \times 100}{4 \times 2.035} \quad +82.3^\circ \pm 0.6^\circ \text{ (in 1.0 N NaOH)}$$

0.3053 gm., dry, in 15.0 cc. of solution

TABLE II  
*Action of Sodium Hydroxide at 25° on Tetrapeptides*

Compound	Dextro-leucyl-dextro-leucyl-glycyl-glycine	Glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine
Experiment No.....	1, 2, 3	4 and 5
Amount of substance used, gm.	0.8957 (1) 0.8957 (2) 0.8957 (3) (0.0025 mol)	0.8287 (4) 0.8287 (5)  (0.0020 mol)
1.0 N NaOH used, cc.	25.0 (1, 2, 3)	20.0 (4, 5)
Action of alkali at 25°, days	8 (1, 2, 3)	8 (4, 5)
Total N in hydrolysate with 20 cc. 20 per cent HCl (36 hrs. at 100°), gm. per 100 cc.	0.6523 (1) 0.6512 (2) 0.6557 (3)	0.5209 (4) 0.3438* (5)
Amino N Total N, per cent	99.8 (1) 99.2 (2) 98.3 (3)	98.3 (?) (4) 99.0 (5)
$\alpha_D^{20}$ (4 dm. tube), degrees	-1.61 (1) -1.58 <sub>1</sub> (2) -1.58 <sub>1</sub> (3)	-1.82 (4) -1.19 (5)
$[\alpha]_D^{20}$ of leucine in solution, degrees	-13.1 <sub>1</sub> (1) -13.0 <sub>6</sub> (2) -12.9 <sub>1</sub> (3)	-12.4 <sub>1</sub> (4) -12.3 <sub>1</sub> (5)

\* The hydrolysate was diluted with 20 per cent HCl before examination.

The reading (correct to  $\pm 0.05^\circ$ ) was made as soon as the field appeared clear. The rotation of this solution (at about  $31^\circ$ ) dropped by 25 per cent in the course of 48 hours because of hydrolysis and racemization. This perhaps explains why Abderhalden and Fleischmann<sup>8</sup> found for glycyl-levo-leucyl-levo-leucyl-levo-leucine  $\alpha_D^{20} = -1.6^\circ$  (1 dm. tube) or  $[\alpha]_D^{20} = -78.63^\circ$ , employing a

solution of 0.2035 gm. of substance in 1.0 N sodium hydroxide, the volume of the solution being 10 cc.

## 2. Action of Sodium Hydroxide on Tetrapeptides (Racemization)

All the data are recorded in Table II. The alkaline solutions of the tetrapeptides prepared were kept for 8 days in a thermostat at 25°. The solutions were then acidified with hydrochloric acid and concentrated to dryness under reduced pressure in the way indicated in a previous paper.<sup>9</sup> The dry residues were then taken

TABLE III  
*Hydrolysis of Tetrapeptides by Hydrochloric Acid at 100°*

Compound	Dextro-leucyl-dextro-leucyl-glycyl-glycine	Glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine
Experiment No.....	6 and 7	8 and 9
Amount of substance used, gm.	0.8957 (6) 0.8957 (7) (0.0025 mol)	0.8287 (8) 0.8287 (9) (0.0020 mol)
Amount of NaCl added, gm.	0.2923	0.2923
Total N in hydrolysate with 20 cc. 20 per cent HCl (36 hrs. at 100°), gm. per 100 cc.	0.6683 (6) 0.6730 (7)	0.5215 (8) 0.5203 (9)
Amino N Total N, per cent	100.0 (6) 98.5 (7)	100 (8) — (9)
$\alpha_D^{25}$ (4 dm. tube), degrees	-2.03 (6) -2.02 (7)	-2.38 (8) -2.35 <sub>s</sub> (9)
$[\alpha]_D^{25}$ of leucine in solution, degrees	-16.2 <sub>s</sub> (6) -16.0 <sub>l</sub> (7)	-16.2 <sub>s</sub> (8) -16.1 <sub>s</sub> (9)

up in 20 cc. of 20 per cent hydrochloric acid. The test-tubes were sealed and immersed for 36 hours in a steam-heated water bath at 100°. The suspensions obtained were filtered in order to remove the sodium chloride that had separated at room temperature. The total nitrogen was determined on 5 cc. and the amino nitrogen

<sup>9</sup> Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **86**, 715 (1930).

on 1 cc. of solution. The loss of optical activity is given in per cent by the ratio

$$\frac{[\alpha_0]_D^t - [\alpha_1]_D^t}{[\alpha_0]} \times 100$$

The values  $[\alpha_0]_D^t$  of the blanks are given in Table III.

### 3. Action of Hydrochloric Acid on Tetrapeptides (Hydrolysis)

The tetrapeptides were hydrolyzed with 20 per cent hydrochloric acid in the presence of sodium chloride in sealed tubes immersed for 36 hours in a steam-heated water bath at 100°. The total nitrogen was determined on 5 cc. and the amino nitrogen on 1 cc. of solution. All the data are recorded in Table III. The values  $[\alpha_0]_D^t$  were thus obtained.

## STUDIES ON KETOSIS

### II. THE COMPARATIVE KETOLYTIC ACTION OF GLUCOSE, GALACTOSE, FRUCTOSE, AND SUCROSE\*

By HARRY J. DEUEL, JR., MARGARET GULICK, AND J. S. BUTTS

(From the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles)

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Shaffer (1) has assumed that all metabolizable monosaccharides are of equal value in serving as precursors of the active intermediary derivative of carbohydrate which possesses ketolytic action. Such an assumption seems logical, if our present conception is correct that the oxidation of the monosaccharides is preceded by their transformation into glucose (either before or after their conversion into glycogen). However, all of the sugars do not have an identical physiological behavior.

According to Lusk (2), fructose exerts the greatest specific dynamic action, followed in order by sucrose, glucose, and galactose. Galactose and fructose, as well as sucrose, are oxidized at a more rapid rate in the human than is glucose or maltose, as judged by the rate of increase of respiratory quotient after their administration (Deuel (3)). Voit (4) believes that glucose and fructose are more satisfactory sources of glycogen than is galactose. In view of these differences it might seem that the sugars would show variable ketolytic actions.

Goldblatt (5) concluded from experiments on fasting human subjects that glucose, fructose, sucrose, and maltose were ketolytic, while galactose, mannose, lactose, and glycerol exerted no such effects. His results seemed entirely at variance with the work of Deuel and Chambers (6), in which it was shown galactose and lactose caused a nitrogen-sparing action when administered to phlorhizinized dogs, a phenomenon which Wierzechowski (7) later demonstrated to occur concomitantly with the decrease in the

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excretion of acetone bodies. Chambers and Deuel (8) also reported that glycerol is a glucose former and produces a protein-sparing action when administered to phlorhizinized dogs.

The present paper reports the study of the antiketogenic activity of sucrose, galactose, and fructose as compared with glucose.

### *General Procedure*

The ketolytic effect of glucose and galactose was studied in normal human subjects in whom a ketosis was produced either by a prolonged protein-fat diet or by a preliminary period of fasting (usually 4 days). The experiments on the antiketogenic action of fructose and sucrose were made only in the fasting subjects. In most cases the urine was divided into three 8 hour samples daily, although in a few instances 12 hour or 24 hour periods were used. Toluene served as a preservative. The routine tests for sugar and albumin on the urine of all the subjects just previous to the experimental periods were found negative in all cases.

Urinary nitrogen was determined by the Kjeldahl method, urine sugar (when present) by the Bertrand procedure, and total urinary acetone bodies by the technique of Van Slyke. The quinhydrone electrode was used in the few cases in which the urine pH was determined. C.P. Pfanstiehl sugars were used in doses varying from 25 to 80 gm. dissolved in water with sufficient lemon juice to give an agreeable flavor.<sup>1</sup> The sugar was taken in most cases at 7.00 a.m. immediately after the closing of the previous 8 or 24 hour period. The subjects for the experiments reported here were, in addition to the authors, graduate students, laboratory technicians, and others closely connected with the medical school who were of unquestioned honesty. All were presumably normal individuals.

## EXPERIMENTAL

### *Experiments on Protein-Fat Diet*

Three experiments were conducted on different subjects in order to study the comparative antiketogenic effect of glucose and

<sup>1</sup> The lemon juice was used in the proportion of 55 gm. to 75 gm. of sugar. The ketolytic effect of the maximum amount of lemon juice was negligible as demonstrated in the control experiments. Moreover, similar amounts were taken by the same subject when the different sugars were taken so that any slight effect would be constant.

galactose. The diet for the first subject (H. J. D.) consisted of bacon, eggs, ground beefsteak, and lettuce, and had a nitrogen content of 14.24 gm. Agar was taken in 10 gm. amounts daily for bulk. The approximate caloric value of the diet, which was uniform throughout the experiment of 33 days, amounted to 1800 calories. This permitted a continual oxidation of body fat for the period during which the diet was continued. The subject's weight gradually fell from 208 pounds at the beginning of the experiment to 183 pounds at the conclusion. The second and third subjects (J. S. B. and P. W. S.) partook of a somewhat more liberal diet in which the monotony observed by the first subject was avoided by a rotation of foods during 3 day periods. The caloric intake of these diets approximated 2300 calories per day and the average nitrogen content for the 3 day periods was 14.76 gm. per day. Although some changes were made in the food during the early part of the experimental period to suit the individual tastes, that used throughout the latter two-thirds was uniform.<sup>2</sup> The caloric intake was insufficient to maintain a constant body weight, that of J. S. B. falling from 152.5 to 139 pounds, while the weight of P. W. S. dropped from 135 to 124.5 pounds. In all cases the subjects remained in good health during the experiment, although it was noted that a considerable lassitude was constantly present. This condition was almost immediately improved by the administration of carbohydrate. This weakness, which was probably partly ascribable to the attendant acidosis, was particularly experienced in climbing stairs or doing other tasks where only moderate physical exertion was necessary. It should also be noted that such a mental condition was experienced that it was practically impossible to concentrate on the usual mental problems.

<sup>2</sup> The diet for J. S. B. and P. W. S. was as follows:

*1st Day*—Breakfast: bacon, eggs, cream, and coffee. Lunch: lettuce, mayonnaise, meat loaf, cream, and coffee. Dinner: spinach, egg, butter, celery, bacon, and beefsteak.

*2nd Day*—Breakfast and lunch as above. Dinner: cucumber, string beans, butter, egg, and pork chops.

*3rd Day*—Breakfast and lunch as above. Dinner: sauerkraut, egg, tomato, bacon, and roast lamb.

The soluble carbohydrates were removed from all of the cooked vegetables by boiling them at least 5 minutes, draining the water, then adding



*Development of a Constant Elimination of Acetone Bodies*—In each case cyclic variations in the excretion of the acetone bodies

TABLE I

*Nitrogen and Acetone Body Excretion during the Early Days of a Protein-Fat Diet*

Day	H. J. D.		J. S. B.		P. W. S.	
	Total N	Total acetone bodies	Total N	Total acetone bodies	Total N	Total acetone bodies
	gm.	gm.	gm.	gm.	gm.	gm.
0	14.02		10.04	0.087	10.12	0.072
1	16.71	0.133	13.26	0.162	16.41	
2	23.38	1.006	19.37	0.692	18.86	0.471
3	21.09	1.095	19.68	1.629	20.20	0.632
4	21.20	2.496	20.41	2.019	19.50	0.975
5	18.86	2.713	18.87	1.830	19.44	0.874
6	16.74	4.893	19.25	2.177	18.21	1.895
7	17.69	6.414	18.10	2.961	19.22	2.182
8	16.55	5.171	18.89	3.298	18.13	2.282
9	16.20	8.234	18.19	2.212	16.70	2.040
10	16.55	6.212	18.52	3.298	18.13	1.390
11	15.77	5.304	16.59	2.282	17.57	1.203
12	16.05	6.308	16.00	3.546	16.35	1.275
13	16.25	7.053	16.23	4.241	16.54	3.050
14	15.97	6.625	15.93	2.579	15.40	1.309
15	15.27	5.258	16.70	2.889	17.89	1.696
16	14.68*	5.729*	16.69	3.100	15.36	3.179
17	14.04	4.452	15.86	3.010	17.12	1.324
18	13.99	5.906	15.78	3.497	16.00	1.905
19	14.23	5.698	16.18	3.993	16.84	1.022
20			15.06	2.207	16.93	0.774
21					15.98	1.804
22					15.49	1.500
23					15.44	1.612
24					15.37	1.408
25					16.67	1.716
26					17.51	0.789
27					16.86	0.923

\* 25 gm. of glucose were taken on this day.

were noted, although fairly constant results were obtained with two of the subjects during the latter part of the experiment. Table I shows the comparative figures for acetone body and

nitrogen excretion during the period prior to the administration of sugar.

The acetone body excretion reaches a fairly constant level with H. J. D. after the 7th day. Somewhat larger variations were noted in the case of the other two subjects. The nitrogen output reaches a very high negative level during the 1st week of the diet in all three experiments. This is to be attributed to the withdrawal of the carbohydrate food with the resulting loss in protein-sparing action. After the 1st week the nitrogen excretion gradually declines, although the intake is constant, and only small negative balances were obtained during the latter part of the experiments. Concomitantly with the increased total acetone elimination, there was a greater urine acidity. With subject H. J. D. this reached a maximum on the 25th day of the diet when the pH was 4.98.

*Diurnal Variation of Acetone Body Excretion*—The hourly excretion of acetone bodies in the urine during each of the 8 hour periods is given in Table II. There is a consistent diurnal variation in the excretion of acetone bodies in the urine. The highest value was obtained from 3 p.m. to 11 p.m., the intermediate amount was found during the overnight interval (11 p.m. to 7 a.m.), and the smallest quantity was excreted during the morning and early afternoon period (7 a.m. to 3 p.m.). This variation is masked during the first days of the diet because of the gradual steady increase in the level of ketosis from day to day. In only three instances in the 14 days reported above does the middle period fail to show the highest rate of excretion, while a like regularity was found with the other periods. Forrsner (9), McQuarrie and Keith (10), and McClellan and Toscani (11) have also found that the excretion of acetone bodies is at a minimum during the morning and early afternoon. However, in Forrsner's experiments, the maximum elimination of these compounds occurred in the night period and it sometimes extended over until the early morning samples. This may have resulted because of the exceptionally high fat content of the evening meal as compared with ours. Hubbard and Wright (12) concluded that the maximum rate at which the acetone bodies were eliminated bore a relationship to the time at which the meals were ingested, although the largest excretion noted in any of his experiments was one-

eighth to one-tenth the minimum quantity which we have found in the experiments here reported.

*Comparative Ketolytic Action of Glucose and Galactose*—Since glucose may be considered the most physiological of the sugars, it is used as a standard with which to compare galactose. We have made comparisons with glucose because it forms the chief, if not the only reducing sugar of the blood, since it is the mono-

TABLE II

*Diurnal Variation in Excretion of Acetone Bodies in Urine during a Protein-Fat Diet*

Subject H. J. D.

Day on diet	Total acetone bodies			
	7 a.m.-3 p.m.	3-11 p.m.	11 p.m.-7 a.m.	24 hrs.
	mg. per hr.	mg. per hr.	mg. per hr.	gm.
6	145.0	296.1	189.4	4.893
7	204.3	357.7	264.3	6.414
8	171.3	229.8	244.4	5.171
9	247.4	400.7	394.5	8.234
10	225.2	275.5	271.1	6.212
11	162.4	260.7	246.4	5.304
12	254.6	267.5	266.2	6.308
13	263.5	295.3	335.3	7.053
14	235.2	278.5	264.5	6.625
15	195.3	241.5	221.1	5.258
16*	142.0	350.3	221.5	5.729
17	154.2	251.3	151.9	4.452
18	321.3	196.8	212.4	5.906
19	163.4	309.6	238.4	5.698
Average.....	209.6	286.5	251.5	

\* 25 gm. of glucose were ingested on this day.

saccharide formed when glycogen is hydrolyzed by acid or by glycogenase and maltase and because it is the sugar which is excreted by diabetics. Moreover, the body is called on to metabolize large amounts of it because of the predominance of starch in the diet. Approximately 75 gm. of these sugars were ingested by H. J. D. after the level of ketonuria had been shown to be constant for several days. J. S. B. received only 50 gm. of each of the monosaccharides, while P. W. S. took only 25 gm. The results are summarized in Table III.

In each of the three subjects, the administration of galactose was followed by a greater ketolytic action than was that of a like amount of glucose, despite the fact that in at least one case some of the former sugar was lost by the resulting galactosuria. The greater antiketogenic effect of galactose was exhibited not only

TABLE III

*Elimination of Acetone Bodies before and after Glucose and Galactose during a Protein-Fat Diet*

Sugar retained		Total acetone bodies								
Kind	Amount	Before sugar	Days after sugar					Minimum level		Resumption of original rate of excretion
			1	2	3	4	5	Period	Amount	
Subject H. J. D.										
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	hrs. after sugar	gm. per 8 hrs.	hrs. after sugar
Glucose	75	5.70	3.20	4.09	6.97	9.48		9-16	0.960	33-40
Galactose	71.2	7.63	2.77	2.32	6.66	10.26	9.07	25-32	0.546	57-64
Subject J. S. B.										
Glucose	50	2.21*	1.54	2.84	3.15	1.81	2.73	1- 8	0.341	25-32
Galactose	50	2.53†	0.77	1.00	1.13	1.48	2.30	13-24	0.201	After 73
Subject P. W. S.										
Galactose	25‡	0.92	0.55	0.46	0.77	0.81		25-36	0.074	73-84
Glucose	25	0.81	0.73§	1.03	1.20	0.62		1-24		25-32

\* The previous days were somewhat variable. The average of 7 days is 3.04.

† The previous days were somewhat variable. The average of 6 days is 2.43 gm.

‡ No qualitative tests were made on urine. This is the maximum amount retained.

§ There was no separation of urine into 12 hour periods on this day.

by the lower values to which the total acetone excretion was depressed, but also by the longer period at which this minimum level was noted.

With H. J. D. the excretion of the acetone bodies was actually lower on the 2nd day after the administration of galactose than

it was on the day on which the sugar was taken, while with glucose the minimum value was reached on the second 8 hour period after its ingestion. The resumption of the original level of excretion of acetone bodies (as determined by the first 8 hour period in which their elimination exceeded that of any period on the day prior to the ingestion of carbohydrate) also occurred 24 hours earlier with glucose than with galactose. Similar though not such striking differences were noted in the experiments on J. S. B. and P. W. S. who took only 50 and 25 gm. of these sugars respectively. These results are all in harmony in indicating that galactose actually does exert a ketolytic action and they suggest that in all probability its action in this respect is more potent than that of glucose. That the discrepancy between these results and those of Goldblatt (5) is not to be attributed to the different method of producing the ketosis is indicated by the experiments reported in the following section in which the ketolytic action of these sugars and others has been compared during a fasting ketosis.

#### *Experiments with Fasting Subjects*

*Excretion of Acetone Bodies*—The ketolytic action of sucrose, fructose, and galactose has been compared with that of glucose during fasting on seven subjects. The sugars were usually administered with lemon juice on the morning of the 5th fast day, although in some cases with the female subjects, it was necessary for them to be taken earlier to counteract the severe acidosis. The ketonuria during the early days of the fast has been reported in a previous paper of the authors (13) in which it was demonstrated that a distinct variation in the extent of acidosis developed exists between the sexes. In the present paper the urinary acetone values are given only for the day preceding the ingestion of the sugar. Table IV gives the summary of the ketolytic action of the four sugars examined.

In the experiments on subjects with ketosis due to fasting, a greater antiketogenic action was also noted after the ingestion of galactose than was found after the administration of a like quantity of glucose. The superior ketolytic action of the former sugar is most pronounced with subjects H. J. D. and R. B. who had the largest doses of these sugars. With H. J. D. the total acetone

TABLE IV

*Ketolytic Effect of Fructose, Galactose, and Sucrose As Compared with Glucose in Fasting Subjects*

Subject	Sugar retained		Total acetone bodies						
	Kind	Amount	Before sugar	Days after sugar			Minimum level		Resumption of original rate of excretion
				1	2	3	Period	Amount	
		gm.	gm.	gm.	gm.	gm.	hrs. after sugar	gm. per 8 hrs.	hrs. after sugar
H. J. D.	Glucose	72.2	7.79	1.37	4.26	7.55	17-24	0.260	41-48
	Galactose*	72.2	8.93	1.43	1.51	5.15	25-32	0.201	65-72
	Fructose†	72.2	8.53	1.05	2.17	7.57	17-24	0.234	49-56
	Fructose	72.2	6.47	1.31	2.07	9.30	9-16	0.236	41-48
	Glucose	72.2	6.83	2.21	4.74	11.82	9-16	0.554	41-48
G. C. D.	Sucrose‡	57.0	20.67	2.79	1.08	0.58	56-72	0.122	After 72
	Glucose	60.0	12.27	2.79	3.64	4.56	1-8	0.473	" 72
R. B.	"	70.0	19.19	7.38	7.08	12.40§			
	Galactose	74.4	14.72	7.51	6.48	5.55	17-24	1.385	After 64
	Sucrose	75.0	12.12	3.96	2.39	11.98¶	25-32	0.386	57-64
P. W. S.	Glucose	25.0	3.48	2.56	7.04	2.78	1-8	0.543	9-16
	Galactose	25.0	3.42	1.57	6.36	3.90	17-24	0.236	25-32
	Sucrose‡	23.8	3.62	1.39	3.53	5.16	17-24	0.307	25-32
	Glucose	25.0	4.84	1.88	5.86	5.45	17-24	0.525	25-32
V. T.	"	58.2	11.29	3.46	4.67	7.43	9-24		
	Galactose	**	7.95	2.86	3.82	9.24	17-24	0.665	57-64
	"	††	9.09	5.79	4.87	7.83	25-32	0.961	33-40
C. T.	Glucose	25.0	6.18	3.79	8.67	8.25	1-8	1.091	25-32
	Fructose	25.0	4.86	3.23	12.41	7.47	1-8	0.960	25-32
J. S. B.	Glucose††	25.0	0.60	0.58	2.07				
	Sucrose‡	23.8	7.51	4.00	8.55	7.63	17-24	0.627	25-32
	"	23.8	7.51	2.67	8.14	12.93	17-24	0.671	25-32

\* 75 gm. of galactose were ingested. 2.8 gm. were excreted in urine.

† There was slight diarrhea shortly after sugar was taken.

‡ The dose was calculated to give monosaccharides equivalent to the glucose ingested.

§ The value was determined by analysis. The excretion for the first 16 hours was 6.43 gm.

|| Estimated value. Acetone for the first 16 hour period was 3.70 gm.

¶ The value was determined by analysis. Excretion for the first 16 hours was 5.87 gm.

\*\* 60 gm. of galactose were ingested. Considerable loss was probable because of diarrhea. 1.81 gm. were excreted in urine.

†† 60 gm. of galactose were ingested. Considerable loss was probable

excretion was depressed to the minimum level for 2 days after galactose while a marked increase in the elimination of acetone bodies had already begun on the 2nd day in both experiments after the administration of the same amount of glucose. With R. B., a female subject, a greater sparing action was obtained on the 3rd day after galactose than on the previous 2 days, while a rise in the excretion of the ketone bodies had already taken place on the 3rd day after glucose. Not only was the ketolytic action prolonged over a longer period following galactose but the minimum level reached with H. J. D. was also lower. The results on P. W. S., who took only 25 gm. of the sugars, are less definite, although a lower ketonuria was obtained on the day of carbohydrate ingestion with galactose than with either of the glucose experiments. The results on the fourth subject (V. T.) are atypical and may be disregarded because she experienced a considerable diarrhea in both galactose experiments, while this was absent in the glucose test with which they are compared.

In both experiments on H. J. D. the ketolytic action of fructose was found to be intermediate between galactose and glucose, while with C. T., who ingested only one-third the amount of sugar that the former subject did, no definite difference is to be noted. In all the five experiments on four subjects, the antiketogenic effect of sucrose would likewise seem to be superior to that of glucose. With J. S. B. a marked ketolytic action followed the ingestion of 23.75 gm. of sucrose, while no response was obtained with 25 gm. of glucose. The small dose of sucrose is almost as effective in both cases with J. S. B. as three times as much glucose in the two experiments on H. J. D. The results on G. C. D. are especially interesting. She developed the most pronounced acidosis experienced in any of our subjects and it was necessary to administer sucrose on the morning of the 4th day. The excretion of ketone bodies progressively declined and was at the lowest level 72 hours later when the experiment was concluded. Decidedly different results were obtained after the ingestion of glucose. Although the ketosis did not reach the level in the 3 days after glucose at which it had been prior to the ingestion of the sugar, there was a progressive rise in the extent of the acetone body elimination on the 2nd and 3rd days after the sugar.

*Control Experiments*—The excretion of acetone bodies gradually

rises during the early days of fasting. It is generally assumed that a level is reached in 4 or 5 days after which it remains fairly constant. In most of the experiments reported here, the sugar was taken on the 5th day when it was assumed that a constant excretion was to be expected had not the carbohydrate been ingested. In several of the experiments with women in which the sugar was not taken until the 5th day and in some of the tests on men (13) a fair uniformity was found between the acetone excretion on the 3rd and 4th fast days which is indicative that a constant *niveau* had been reached. In those cases in which a somewhat greater elimination of the acetone bodies was noted on the 4th fast day than was found on the 3rd one, the increase was only slight when compared with the variation between the 2nd and 3rd

TABLE V  
*Excretion of Acetone Bodies in Control Experiments on Fasting*

Subject	Fast No.	Acetonuria on fasting day							
		Control	1st	2nd	3rd	4th	5th*	6th	7th
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
J. S. B.	5		0.084	1.494	4.436	7.436	7.759	10.170	
H. J. D.	6	0.064	0.295	5.168	7.283	8.952	6.640	9.518	7.595

\* Lemon juice was taken without other sugar on this morning.

fast days. We feel that we are justified in comparing the ketolytic action of the sugars on the assumption that the decreases in ketonuria experienced after their ingestion are proportional to their ketolytic activity. It also seems fair to assume that such a drop in the elimination of the acetone bodies is not adventitious and would not have occurred had not the sugar been ingested. The results of two control experiments on two subjects are given in Table V.

Essentially constant results were obtained on H. J. D. from the 4th to the 7th fast days when an amount of lemon juice equal to that taken in the experiments with the sugars was ingested on the 5th day. A slight decrease in the excretion of acetone bodies occurred on the 5th day which is probably to be traced to the antiketogenic effect of the sugar in the lemon juice. This decline, however, is slight when compared with that obtained after the



ingestion of 72.2 gm. of the sugars. The results on J. S. B. are slightly different in showing no decrease in acetonuria on the 5th day but the quantity of lemon juice taken by this subject was less than in the other control experiment. No control experiments on women were possible because of the severity of the acidosis found in every case (13).

*Nitrogen Excretion*—In a former paper (13) it was demonstrated, in harmony with many earlier investigations of other observers, that the urinary nitrogen decreased from the control level on the 1st and sometimes on the 2nd fast day because of the sparing effect of the body glycogen on the protein metabolism. This was followed by a rise in nitrogen excretion on the 2nd or 3rd fast day which occurred concomitantly with the appearance of an appreciable excretion of total acetone bodies. The maximum level was reached not later than the 3rd fast day, after which a second gradual decline in urinary nitrogen occurred. When the sugars were ingested, there was an abrupt drop in the output of nitrogen due to the sparing action of the carbohydrate. The nitrogen elimination increased after the sparing action of the sugar was spent but it usually did not return to the original value found on the 4th fast day because of the normal gradual decrease which would have taken place if the sugar had not been ingested. Table VI gives an analysis of the data showing the comparative nitrogen values after the different sugars were ingested.

In four of the five experiments galactose seems to exert a greater protein-sparing action than does glucose. In most cases these results are parallel with those on the decrease of ketone body excretion shown in Table IV. With the exception of P. W. S. who ingested the smallest amounts of these sugars, the reduction of the urinary nitrogen persists for a longer period after galactose than after glucose; a lower minimum value is reached and this at a later period. Such differences are even noted with V. T. in whom there was no distinct superiority in ketolytic action of galactose (probably because of the decreased absorption of the sugar as the result of a diarrhea). A superior protein-sparing action exists with fructose in one test on H. J. D. and in the experiment on C. T. The results with sucrose are not as consistent, although in three of the five experiments the urinary nitrogen is undoubtedly lower after sucrose than after glucose.

TABLE VI

*Urinary Nitrogen before and after Administration of Fructose, Galactose, and Sucrose as Compared with Glucose*

Subject	Sugar retained		Before sugar	Days after sugar			Minimum N excretion		Resumption of original N excretion
	Kind	Amount		1	2	3	Period	Amount	
		gm.							
H. J. D.	Glucose	72.2	12.21	8.08	10.05	9.70	17-24	2.07	25-32
	Galactose	72.2	11.97	7.76	7.21	9.35	25-32	1.87	After 73
	Fructose	72.2	11.20	6.93	7.79	10.13	9-16	2.15	49-56
	"	72.2	11.45	8.34	7.92	10.33	9-16	2.13	41-48
G. C. D.	Glucose	72.2	11.72	8.48	8.67	10.59	9-16	2.27	49-54
	Sucrose	57.0	9.44	7.33	7.45	8.12	25-32	2.19	41-48
	Glucose	60.0	8.06	5.86	6.21	7.08	17-24	1.83	After 73
R. B.	"	70.0	9.89	7.31	6.50	8.58			
	Galactose	74.4	10.66	8.34	6.81	6.36*			
	Sucrose	75.0	13.63	7.78	9.50	11.24	9-16	1.94	65-72
P. W. S.	Glucose	25.0	11.63	10.24	10.90	9.37			
	Galactose	25.0	11.54	9.96	10.39	9.89			
	Sucrose	23.8	11.80	9.80	9.85	10.42			
	Glucose	25.0	11.91	8.67	9.85	9.15			
V. T.	"	58.2	9.00	6.54	6.95	7.74	9-24	1.88	
	Galactose	†	8.09	5.78	5.81	5.97	17-24	1.25	
	"	†	9.26	7.19	5.60	6.99	41-48	1.55	
C. T.	Glucose	25.0	14.61	13.65	10.65	11.02			
	Fructose	25.0	15.52	12.83	12.31	11.86			
J. S. B.	Glucose	25.0	11.97	9.56	10.40				
	Sucrose	23.8	10.86	10.12	10.50	9.75			
	"	23.8	12.70	10.04	9.95	10.13			

\* This value was calculated from the 16 hour period.

† Considerable diarrhea.

TABLE VII

*Urinary Nitrogen in Control Fasting Experiments*

Subject	Lemon juice	Fast No.	Urinary N per day during fasting							
			Control	1st	2nd	3rd	4th	5th	6th	7th
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
J. S. B.	20	5	13.59	10.86	11.70	12.74	13.73	11.90	11.00	
H. J. D.	55	6	11.64	10.26	11.84	11.80	12.39	10.90	11.91	10.46

Since the excretion of nitrogen in the urine is much more regular than that of the acetone bodies, it would seem that these experiments might offer more cogent proof of the superiority of galactose over glucose, and to a less extent that of fructose and sucrose over glucose. These results are especially of importance in showing that the superiority of these sugars would seem to be a real one and not to be traced to a greater loss of the acetone from the lungs (with a consequently lower excretion in the urine) because similar differences were observed on nitrogen excretion for which the sole pathway of elimination is by way of the kidneys.

The nitrogen excretion of the control experiments in which no carbohydrate (other than that in the lemon juice) was taken is shown in Table VII.

Although there is a small drop in the urinary nitrogen on the 5th day following the ingestion of the lemon juice, these variations are slight compared with the decrease which followed the ingestion of the various sugars. The sparing action which is noted in Table VI must be considered a real one rather than an accidental effect.

#### DISCUSSION

If any differences were to be observed in the ketolytic behavior of the various sugars, one would anticipate that glucose would be the most effective and that the other sugars would vary in this property according to their ability to be changed to this monosaccharide. However, we were much surprised to find that glucose was actually inferior as an antiketogenic agent in the majority of instances when compared with galactose, fructose, or sucrose. In no case was glucose found to be definitely superior to any of the other sugars.

In general, the results with galactose show the most marked superiority over glucose of the three sugars investigated. This variability was unexpected in view of the fact that galactose is a more difficultly metabolized sugar than fructose, sucrose, or glucose, as evidenced by the greater readiness with which a hyperglycemia and a melituria may be produced by it (14, 15). Likewise, it has usually been considered that it is a very poor glycogen former (15, 16). Moreover, galactose along with lactose, mannose, and glycerol has been classed by Goldblatt (5) as a substance

without antiketogenic action in contrast with the positive results which this investigator found on glucose, sucrose, maltose, and fructose. We are of the opinion, however, that no conclusions can be drawn from the work of Goldblatt, since his observations are based on *qualitative tests* rather than on *quantitative determinations* of the acetone bodies. The number of his subjects is also limited. In addition there is some question as to whether his method of producing ketosis by a 36 hour fast of food and water gives a sufficiently uniform level from which to start the experiments.

The better effect of galactose was exhibited by the greater and more prolonged decrease in the elimination of the acetone bodies in the period following its ingestion. One might suggest that the 8 hour periods into which the urine was separated are too long to demonstrate the full effect of the glucose. However, the same criticism may equally well apply to galactose. It does not seem reasonable to assume that during such a short period a more pronounced effect could be shown by glucose than by galactose, especially when one remembers that the ketonuria during the period of maximum ketolytic activity is usually lower after the latter sugar. Such differences were noted not only in the experiments in which the ketosis was produced by fasting but also in those tests where a constant ketonuria was obtained by a uniform protein-fat diet.

Since the excretion of acetone from the lungs is small, as shown by Folin and Denis (17), we have not attempted to correct for it. That the superior ketolytic effect of galactose is real and not to be attributed to a greater loss of this constituent from the lungs (with the consequent lessened excretion through the kidneys) after this sugar than after glucose, is indicated by the greater nitrogen-sparing action which it exhibited over that of the latter sugar. There is a considerable parallelism between the decrease in urinary nitrogen values and the drop in ketonuria. In the galactose experiments the minimum level of nitrogen elimination is lower and it occurs longer after the ingestion of the sugar than is the case in the tests with glucose. The period of noticeable nitrogen-sparing action is usually more prolonged with the former sugar.

The results on fructose seem to indicate a similar although

probably not as marked superiority over glucose. It is impossible on the basis of our results to compare quantitatively galactose, fructose, and sucrose in this respect, since experiments on all of the sugars were not made on a single subject. With H. J. D., who showed quite consistent results throughout the series of tests, fructose was found to stand intermediate between glucose and galactose. With R. B. sucrose was found to be better than glucose but its action was not as prolonged as that of galactose. We feel that the absolute comparisons of the ketolytic action of the sugars can be more satisfactorily ascertained by a study of the nitrogen-sparing action of the carbohydrates on fasting animals which do not show an appreciable fasting ketosis than they can be by the more tedious procedure of using fasting human subjects. Experiments are now in progress with fasting rats in this laboratory to study the comparative protein-sparing action of the various carbohydrates.

In many cases in which a ketolytic action was demonstrated on the 1st or the 1st and 2nd days following the ingestion of the carbohydrate, a secondary compensatory increase in ketonuria occurred immediately thereafter, so that the level of the acetone far exceeded that of the control day. In the experiments with H. J. D. on a protein-fat diet, this rise was inversely proportional to the previous decline occasioned by taking the sugar. This was found not only in the two experiments reported in this paper but in another one in which lactose was given. If one averages the total acetone excretion for the 3 or 4 days after the sugar, it would appear that no ketolytic action had been produced because the drop in ketonuria would be masked by the later rise. Although we have not made studies on the concentration of acetone bodies in the blood to show the fall in ketosis after the ingestion of the sugars, we are convinced that the primary decline is to be traced to an actual decreased production rather than to a delayed excretion. Otherwise, we would have no adequate explanation of the nitrogen-sparing action or the decreased acidity of the urine which occurs concomitantly with the decline in ketonuria. Moreover, the ketone bodies would have to be stored in the blood or tissues for 4 days in the galactose experiment on H. J. D. before the excess was excreted. This seems entirely improbable in view of the marked improvement in the subjective feeling which always

followed the ingestion of the sugar and which could not have occurred had there been an increased store of acetone bodies in the blood and tissues. Such a secondary rise was also observed in some of the experiments on the fasting subjects. That this is also a temporary effect is shown by at least five experiments in which the large increase in ketonuria on the 2nd day after the administration of the sugar is followed by a return to the normal level on the 3rd day following its ingestion (Experiments 1, 2, and 4 on P. W. S. and both on C. T.). In those experiments in which the ketolytic action persisted to the 2nd day, it is probable that the high level found on the 3rd day after taking the sugar would have been followed by a substantially lower one on the following day. We have no explanation for this result other than that as the fast progresses the disposal of the ketone bodies may be carried on more efficiently. There is some indication that this may be the case for it has been noted that in a number of experiments the ketonuria reaches a peak on the 3rd day of fasting and that the level falls on the 4th one even without the administration of any food (13). This efficiency may be temporarily lost when carbohydrates are taken in large amounts, only to be regained several days after they have been metabolized.

In order to account for the superior behavior of galactose, one must assume that the blood sugar and the carbohydrate store in the tissues are maintained at a high level for a longer period than after glucose. In one subject (R. B.) the blood sugar was 65 mg. per 100 cc. on the day before galactose (4th fast day); it had risen to 111 on the 2nd day (6th fast day) and it was still at 93 on the 3rd day after the sugar. The comparative results on glucose were 73, 100, and 70 mg. per 100 cc. While we realize that it is impossible to draw any conclusions from a single experiment, we are including these data in support of the view that the superior ketolytic behavior of galactose must be bound up with a more prolonged carbohydrate store. It is difficult to see how it could be retained for this length of time as the original sugar, inasmuch as the ketolytic action reached its maximum 25 to 32 hours after its ingestion and the effect was still noticeable on the 3rd day. However, we have some evidence that the muscle sugar reaches a much higher level in rats after galactose than after glucose and that this difference may be evident even after 24 hours (18).

It is also possible that galactose may be a more efficient glycogen former than is now recognized. Although we have no data as to ability of the human to form glycogen from this carbohydrate, we have found considerable amounts may be deposited in the livers of fasting dogs after the administration of moderate amounts of this sugar. In some unpublished experiments (19) on these animals we have obtained as high as 5.77 per cent liver glycogen 12 hours after the administration of galactose while the results on glucose controls are much lower. Moreover, we have found that the residual liver and muscle glycogen in rats previously fed on a galactose diet is more than twice as high as that of glucose controls after 32 and 56 hours of fasting (18). The absolute proof of the superior glycogenic ability of galactose involves the demonstration that the glycogen is also at higher levels at various shorter periods of fasting after galactose than after glucose.

#### SUMMARY

Galactose has been demonstrated to possess a greater ketolytic action than glucose in the human both on ketosis due to fasting and on that produced by an exclusive protein-fat diet. This superiority is exhibited not only in the larger drop in ketonuria but also in the more prolonged decrease that results. Likewise, there is some evidence to indicate that a more pronounced nitrogen-sparing action follows the ingestion of galactose.

Fructose and sucrose also exhibited a better effect on ketosis due to fasting than did glucose, although our data are insufficient to enable us to make a quantitative comparison between these sugars.

A diurnal variation in the ketonuria was found during the protein-fat diet. The late afternoon and evening period (3 to 11 p.m.) was found to be the highest, while the morning and early afternoon (7 a.m. to 3 p.m.) was the lowest, and the night period was intermediate (11 p.m. to 7 a.m.).

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## OSMOTIC PRESSURE, MOLECULAR WEIGHT, AND STABILITY OF SERUM ALBUMIN\*

By NORVAL F. BURK†

*(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston, and the Department of Physiology, Yale University, New Haven)*

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Because of the discrepancies at the time this investigation was begun in the estimates of the molecular weight of serum albumin, as made by Sørensen (1) (45,000) and Adair (2) (62,000) by means of osmotic pressure experiments, and by Svedberg and Sjögren (3) (67,500) from ultracentrifugal measurements, the osmotic pressure of this protein was reinvestigated under more suitable experimental conditions both in aqueous solution and certain mixed solvents, especially urea solutions. The molecular weight estimates of serum albumin reported here are in good agreement with those given in the more recent paper of Adair and Robinson (4), which became available shortly after the completion of the major portion of the experimental part of this paper.

Serum albumin is denatured by urea. Comparison of osmotic pressure measurements in aqueous and in urea solutions under similar experimental conditions makes it possible to consider whether changes in molecular weight accompany the process of denaturation of this protein. The recent investigations of Hopkins (5) have shown that the denaturation of certain proteins by urea is accompanied by changes in the sulfur linkages of the protein molecule.

\* Part of this material was presented before the American Society of Biological Chemists at Montreal, April 10, 1931.

† National Research Council Fellow in Biochemistry, 1929-31, during which time this investigation was in progress.

## EXPERIMENTAL

The osmotic pressure measurements were carried out upon solutions of serum albumin contained in collodion sacs, which were fitted to glass manometer tubes to form a pendulum type of osmometer, as described in a previous paper (6). The solutions were stirred by moving the osmometers back and forth in a thermostat by means of a Warburg type of apparatus. For the measurements at 0°, an ice bath was used, which was kept in a cold room at approximately this temperature. After 1 or 2 weeks the manometer heights, which were recorded daily, remained constant over fairly long periods, 2 weeks or more in certain test cases. When constancy of pressure was reached, the osmometers were taken apart, the inner solutions analyzed for protein concentration, and the outer solutions qualitatively tested with tannic acid for the presence or absence of protein. In most experiments the tests were completely negative, showing that the membranes used were strictly semipermeable and that protein products of low molecular weight were not appreciably present in the solutions. In cases in which protein was found, the amount was small; less than 1 part in 10,000 as roughly estimated by comparison of turbidities with tannic acid against solutions of known protein content. This error was considered to be negligible.

The albumin was prepared from horse serum by the usual method, following in general the form of procedure given by Svedberg and Sjögren (3). It was crystallized but once in view of the statements of these authors that serum albumin so prepared is homogeneous in mass, but that prepared by recrystallizing several times is a mixture. The crystals were kept at 0° under one-half saturated ammonium sulfate. At various intervals, solutions free from sulfate were prepared from this material by dialyzing in collodion membranes, with stirring, against distilled water in the cold, until the specific conductivity fell below a value of  $10 \times 10^{-5}$  reciprocal ohms.<sup>1</sup> The solutions were then electrodialyzed. The specific conductivity after electrodialysis varied from 1 to  $3 \times 10^{-5}$

<sup>1</sup> During the first stages of dialysis, the insoluble material associated with once crystallized serum albumin, which Hardy and Gardiner (7) and Young (8) consider to be of a lipid nature, separates. It was removed by centrifuging and the dialysis continued upon clear solutions.

reciprocal ohms (protein concentration, about 5 per cent). Toluene was used as a preservative. It was removed from the solutions just before their use in osmotic experiments by passing nitrogen gas into them.

In urea solution, the concentration of serum albumin was determined colorimetrically, as described in a previous paper (6). The standards used in this work were prepared from a pure serum albumin solution of negligible salt content, whose protein concentration was determined from the weight of the residue after drying a known volume of sample to constant weight at 105°. The concentration of serum albumin in aqueous solution was determined either (1) colorimetrically or (2) by drying a sample at 105° and correcting the weight of the residue for the amount of salt in the sample as determined by the composition of the buffer used. The two methods gave concordant results. The latter method was used only for solutions of high protein content. For converting the concentration as obtained from the analysis and expressed in gm. per 100 cc. of solution to that expressed in gm. per 100 cc. of solvent, the value 0.745 (3) was used for the partial specific volume of serum albumin. Owing to limited material, the densities of the protein solutions were not measured, but were calculated from the density of the solvent,  $d$ , and the partial specific volume of the protein,  $\alpha$ , by means of the theoretical relation,  $D = d + \frac{(1 - \alpha d)}{100} C$ , where  $D$  is the density of the solution and  $C$  the concentration in gm. per 100 cc. of solution. The density measurements of Adair (9) on hemoglobin solutions show constancy of  $\alpha$  with respect to protein concentration practically up to saturation (34 per cent).

The capillary rise was measured in dilute solution of protein only. The percentage error introduced by neglecting its variation with protein concentration in capillary tubing of the size used was assumed to be not of significance.

The data of Cohn (10) were used in the preparation of aqueous solutions of acetate and phosphate buffers of a given pH value. For the preparation of urea solutions of known pH, the dissociation-titration curves of these buffers in 6.66 M urea, as previously given (6) and slightly revised, were employed. In most cases, however, the reported pH values were obtained from electrometric

measurements with a hydrogen or quinhydrone electrode in the outer solutions at the end of an osmotic pressure experiment, by using a saturated KCl junction and one of several concordant 0.1 N KCl (or saturated) calomel cells. The temperature was not controlled, but was accurately measured, and the pH values were calculated according to Clark (11), the standard being Sørensen's original value of 0.3380 volt for the 0.1 N calomel cell at 18°.

*Osmotic Pressure and Molecular Weight of Serum Albumin in Aqueous Solution*

The osmotic pressures of solutions of crystalline serum albumin of increasing protein concentration were measured at pH 4.8, dilute acetate buffer being employed for the maintenance of this reaction. The data are given in Table I. Over the concentration range studied, the osmotic pressure is proportional to the protein concentration which has been expressed in gm. per 100 cc. of solvent. Aqueous isoelectric solutions of serum albumin thus conform to the ideal solution law, which for substances of high molecular weight is identical with Morse's modification of van't Hoff's law,  $PV_0 = RT$ , where  $V_0$  is the volume of pure solvent per mol of solute. In this respect, solutions of serum albumin show an osmotic behavior like that of egg albumin (12, 13) under similar experimental conditions, but different from that of the globulin-like proteins, hemoglobin (14), serum globulin (4), and gelatin (12), whose isoelectric osmotic pressure increases to more than is proportional to increases in protein concentration. The linear relation of pressure to concentration permits a direct calculation of the molecular weight of serum albumin by Equation 1, equivalent to the general expression given above,

$$M = \frac{C}{P} RT \quad (1)$$

where

$M$  = molecular weight in gm. of dry protein

$P$  = osmotic pressure in cm. of  $H_2O$  of density 1

$C$  = concentration of dry protein in gm. per 100 cc. of solvent

$RT$  = gas constant  $\times$  absolute temperature,  $22.414 \times 76 \times 13.596 \times 10 = 2.315 \times 10^5$  (100 cc.  $\times$  cm. of  $H_2O$  per gm. molecule at 0°)

Values for the molecular weight calculated at each concentration by Equation 1 are given in the seventh column of Table I. The mean value for the molecular weight, or the mean molecular weight in case the protein in question is a mixture, is 74,600. This value from aqueous isoelectric measurements agrees fairly well with that recently obtained by Adair and Robinson,  $72,000 \pm 3000$  from measurements in alkaline serum albumin solutions (pH 7.4).

TABLE I

*Molecular Weight of Serum Albumin from Measurements of Its Osmotic Pressure in Aqueous Isoelectric Solutions*

Buffer, acetic acid-sodium acetate (0.05 M in total acetate); pH 4.8; capillary correction, 0.80 cm.; temperature 0°.

Experiment No.	C Concentration per 100 cc. solvent	Initial pressure setting*	Equilibrium pressure	P† Osmotic pressure	$\frac{P}{C}$	M Molecular weight
	gm.	cm. solution	cm. solution	cm. H <sub>2</sub> O		gm.
4	0.78	4.5	3.18	2.39	3.06	75,600
24	1.25	4.2	4.78	4.01	3.20	72,400
34	2.79	9.5	9.35	8.53	3.06	75,700
25	2.82	7.7	9.40	8.68	3.08	75,200
9	3.38	16.8	11.49	10.80	3.19	72,600
8	3.49	10.9	11.60	10.91	3.13	74,000
26	4.18	14.0	13.63	13.01	3.11	74,400
82	8.98		28.00	27.84	3.10	74,500
43	12.45	25.0	37.35	37.69	3.02	76,600
Mean.....						74,600

\* Comparison of the figures in this column with those in the fourth column shows equilibrium was obtained starting from either higher or lower pressure.

† The figures in this column are obtained from the previous one by subtracting the capillary rise and multiplying by the density of the solution.

#### *Osmotic Pressure in Ammonium Sulfate Solutions*

The above figure for the molecular weight of serum albumin obtained from measurements in aqueous buffer solution is some 70 per cent greater than that estimated by Sørensen (1) from his osmotic pressure measurements in ammonium sulfate solutions. Besides differences in experimental conditions, our material was, as previously mentioned, once crystallized, while that of Sørensen

was several times crystallized. Svedberg and Sjögren (3) state that serum albumin is a very unstable protein, easily decomposed during the process of purification into products of low molecular weight. They find that serum albumin once crystallized from ammonium sulfate solution is homogeneous with regard to molecular weight, but repeated crystallization by means of this salt produces a mixture which may contain decomposition products to the extent of 40 per cent or more. On the basis of their results they explain the discrepancy between their value for the molecular weight of serum albumin and that obtained by Sørensen by assuming Sørensen's preparations to have contained, as a result of elaborate purification, decomposed protein, giving rise to high osmotic pressures.

It therefore seemed desirable, as a means of further clarification of the question, to investigate the osmotic behavior of our preparations, once crystallized and purified by Svedberg's procedure, in the presence of ammonium sulfate.

A 0.74 N solution of  $(\text{NH}_4)_2\text{SO}_4$  was used as solvent, and as in previous experiments, dilute acetate buffer was employed to maintain the pH in the region of the isoelectric point.

The measurements on solutions of increasing protein concentration are shown graphically as the squares in the upper curve of Fig. 1. The upper three points<sup>2</sup> of this curve are the mean measurements of Sørensen, which have been calculated to 0° for the purpose of comparison. Our measurements confirm those of Sørensen and this agreement would therefore indicate stability of this protein towards repeated recrystallization with ammonium sulfate. This is in complete accord with the results of Adair and Robinson.

Fig. 1 shows that, in the presence of moderately high concentrations of salt, the osmotic pressure of serum albumin increases to an extent that is more than proportional to increases in protein concentration. It is thus evident that the high osmotic pressures obtained by Sørensen, leading to a low molecular weight estimate, can be ascribed to deviations of the osmotic pressure from the ideal solution law, owing to the high concentrations of protein and salt in his experiments, and not to a decomposition of the protein as suggested by Svedberg.

<sup>2</sup> These points are taken from the smooth curves drawn by Sørensen through his data.

*Molecular Weight in Ammonium Sulfate Solution*

Methods for the estimation of molecular weights of proteins in non-ideal solutions have been recently developed by Kunitz (12), Ostwald (15), Burk and Greenberg (6), and Adair and Robinson (4). In general, two modes of procedure have been employed; those which involve extrapolation to infinite dilution, and those which involve correction to proportionality or ideality. The former yield a mean value for the molecular weight and the latter allow comparison of values at each protein concentration, which is of advantage when different preparations are under consideration. In this work a correction method has been employed which gives results practically identical with those obtained by extrapolation.

In Table II are given the data for the osmotic pressure of serum albumin in ammonium sulfate solution. A plot of  $P$  against  $C$  (Fig. 1) shows that the ideal law is not followed except possibly at very low concentrations. To make the data at higher concentrations fit the ideal law it is necessary to find a corrected concentration  $C_0$ , such that

$$P = \frac{RT}{M} C_0 \quad (2)$$

If an empirical relation between pressure and concentration can be found, it would furnish a means of getting  $C_0$  from  $C$ . We find on plotting  $\frac{P}{C}$  against  $C$  (Fig. 2) that the points fall fairly well on a straight line. Hence,

$$\frac{P}{C} = K + BC \quad (3)$$

or,

$$P = KC + BC^2 \quad (4)^*$$

---

\* It is of interest to note that this equation, except for a difference in units, is equivalent to a modification of the van't Hoff equation, for by introducing  $K = 10 \frac{RT}{M}$ ,  $C = \frac{M}{10V_0}$ , and  $a = \frac{BM^2}{100}$  there is obtained  $PV_0 =$

$RT + \frac{a}{V_0}$  where  $a$  is a constant. (The factor 10 is included in the expression

$K = \frac{RT}{M}$  in order to express the constant  $R$  in units of liters.)



where the constant  $K$ , from Fig. 2, is the value of  $\frac{P}{C}$  at infinite dilution (zero concentration) of protein and the constant  $B$ , the slope of the line. Since at infinite dilution, substances conform to the ideal law, it follows from Equations 1 and 3 that

$$K \left( \frac{P}{C} \right)_0 = \frac{RT}{M} \quad (5)$$

TABLE II

*Osmotic Pressure and Molecular Weight of Serum Albumin in 0.74 N (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Solution at 0°*

pH about 4.8.

Experiment No.	$C$ Concentration per 100 cc. solvent	$P$ Osmotic pressure	$g$ Osmotic coefficient	$C_0^*$ Corrected concentration per 100 cc. solvent	$\frac{P}{C_0}$	$M$ Molecular weight
	gm.	cm. H <sub>2</sub> O		gm.		gm.
91	1.88	6.09	1.07	2.02	3.01	76,900
90	2.47	8.39	1.10	2.71	3.08	75,200
89	3.90	13.37	1.15	4.49	2.97	77,900
79	4.13	14.98	1.16	4.80	3.12	74,200
92	5.06	18.12	1.20	6.10	2.97	77,900
53	8.58	35.20	1.34	11.43	3.07	75,400
Mean.....						76,200
Measurements of Sørensen	8.98	36.35	1.36	12.22	2.98	77,700
	13.48	62.80	1.52	20.59	3.05	75,800
	22.46	129.00	1.88	42.30	3.05	75,800
Mean.....						76,600

\* Calculated from the equation  $C_0 = \frac{PC}{P - BC}$ , where  $B = 0.1198$ .

From Equations 3 and 5,

$$\frac{P}{C} = \frac{RT}{M} + BC \quad (6)$$

or,

$$\frac{RT}{M} = \frac{P}{C} - BC \quad (6, a)$$

From Equation 2 is obtained the equation

$$\frac{RT}{M} = \frac{P}{C_0} \quad (2, a)$$

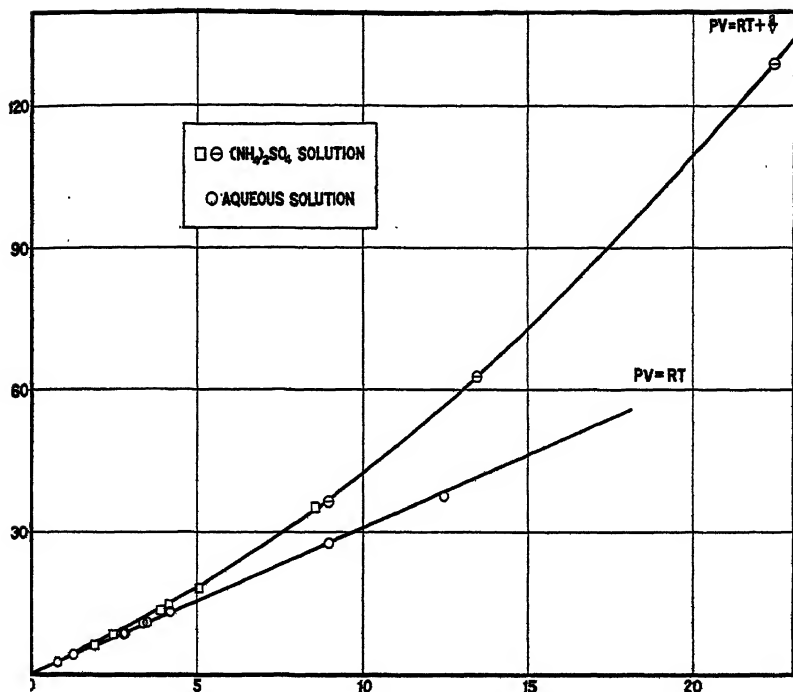


FIG. 1. Relationship of osmotic pressure to concentration of serum albumin, showing deviation of the osmotic pressure from the ideal law in ammonium sulfate solution. Upper curve: solvent, ammonium sulfate solution, 0.74 N; lower curve: solvent, water (0.05 N acetate buffer solution). The ordinate represents the osmotic pressure measured in cm. of water; the abscissa, the concentration in gm. per 100 cc. of solvent.

and from Equations 6, *a* and 2, *a*

$$\frac{P}{C_0} - \frac{P}{C} = BC \quad (7)$$

Equation 7 has been derived from the empirical relation between

$\frac{1}{C}$  and  $C$  (Equation 3) and the assumption that the ideal equation (Equation 1) applies to infinitely dilute solutions. Values of  $\frac{P}{C_0}$  calculated from Equation 7 with the value of  $B$  obtained graphically in Fig. 2, are given in the sixth column of Table II. The fact that they show no trend with changes in concentration indicates that a correct empirical relation between  $C$  and  $C_0$  is given by Equation 7, and that the values of  $C_0$ , or of  $\frac{P}{C_0}$ , so obtained may be used with some confidence in Equation 2 for the calculation of  $M$ . Values of  $M$  so obtained from these data, as well as those of

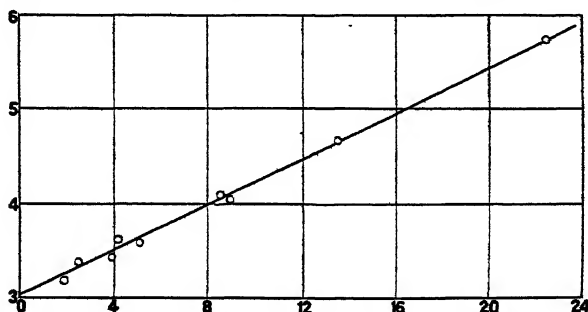


FIG. 2. The osmotic pressure-concentration ratio for serum albumin in ammonium sulfate solution (ordinate) is plotted against the protein concentration which is measured in gm. per 100 cc. of solvent (abscissa).

Sørensen, are given in the last columns of Table II. The mean value for the molecular weight of serum albumin in ammonium sulfate solution is 76,400.

The empirical relation between  $P$  and  $C$ , or between  $C_0$  and  $C$ , described by Equations 3 and 7 may be interpreted in terms of a variable solvation of the protein in ammonium sulfate solutions. The nature of this variation may be seen by rewriting Equation 7 as

$$1 - B \frac{C}{P} C = 100 - 100 B \frac{C}{P} C \quad (7, a)$$

If the protein in these solutions were solvated, the true concentration of the protein in the free solvent would be given by the relation

$$C_0 = \frac{100 C}{100 - hC} \quad (8)$$

in which  $h$  would be the number of cc. of solvent bound to each gm. of protein. In these experiments  $h$  corresponds to the expression  $100 B \frac{C}{P}$  which is not a constant. Since  $\frac{P}{C}$  decreases with decrease in protein concentration,  $\frac{C}{P}$  and therefore  $100 B \frac{C}{P}$  must increase. Its limit, at infinite dilution of protein, would therefore yield a maximum value for the degree of solvation of the protein, if deviation from ideality is ascribed entirely to this cause. Thus we have

$$h_0 = 100 B \frac{C}{P_0} = 100 \frac{B}{K}$$

where  $h_0$ , termed the apparent degree of solvation (or hydration) of the protein at infinite dilution, may be obtained graphically by dividing the slope of line ( $\times 100$ ) by its intercept (Fig. 3). For serum albumin in ammonium sulfate solution  $h_0 = 3.92$  in cc. of solvent per gm. of protein.

The experiments may, therefore, be interpreted on the assumption that the ideal osmotic pressure law holds for a true or corrected concentration of protein in free solvent, the amount of bound solvent decreasing as the protein concentration is increased.

The same experiments may also be expressed, without any assumption of solvation, by the relation given by Hückel (16) for the osmotic pressure of non-ideal solutions, which is

$$P = \frac{RT}{M} gC \quad (9)$$

Here the factor  $g$  is the variable osmotic coefficient introduced simply to express deviations of experimental data, in terms of actual concentrations, from the ideal law, Equation 1. By comparing Equations 2 and 9, it is evident that  $g$  is equal to  $\frac{C_0}{C}$ , and therefore  $\frac{P}{P_0}$ , where  $P_0$  is the ideal osmotic pressure. Equation 7,

therefore, gives an empirical expression for the variations of the osmotic coefficient  $g$  with concentration, which is

$$g = \frac{C_0}{C} = \frac{P}{P - BC^2} \quad (10)$$

or, by eliminating  $P$  by Equation 3

$$g = \frac{C_0}{C} = 1 + \frac{B}{K} C \quad (11)$$

Equation 11 does not suggest any picture of the factor which prevents the data from following the ideal law, nor does it imply anything as to the existence of solvation. It is simply an expression of the empirical fact that these data can be brought into accord with the ideal law by assuming an osmotic coefficient which is equal to 1 at infinite dilution and increases as a linear function of the concentration of protein.

#### *Molecular Weight in Glycerol Solution*

As a further means of testing the stability of serum albumin, a few measurements of osmotic pressure were carried out in a 75 per cent glycerol solution.

Proteins have often been regarded as colloids. It might therefore be supposed that the molecules of this protein possess a certain tendency or capacity to form dispersion (decomposition) or aggregation products when in solution. If such changes are capable of taking place more or less at will, alteration in the nature of the medium ought to alter a given equilibrium condition.

Owing to its high viscosity, pure glycerol could not be used. 75 per cent glycerol does not denature serum albumin, and solutions of this protein in it remain sterile.

From the two measurements made, the molecular weight was found to be 74,700 (Table III). The molecular weight was calculated from the ideal law. This is probably justifiable, since the protein concentration is not very high, and since glycerol, like water, is a non-ionized and non-denaturing solvent. The value obtained is very close to the value for the mean molecular weight in aqueous solution, indicating stability of this protein towards changes in size under conditions which do not affect the protein chemically.

*Molecular Weight from Analytical Data*

Folin and Marenzi (17) have recently determined the tryptophane content of serum albumin. The average of their values is 0.53 per cent. This figure leads to a value, calculated by the procedure of Cohn *et al.* (18), of 78,000 for the probable molecular weight of serum albumin, if it contains 2 tryptophane molecules.

*Osmotic Pressure and Molecular Weight of Serum Albumin in Urea Solution*

The osmotic pressure of this protein in urea solution was investigated primarily to determine the influence of denaturation on its

TABLE III

*Osmotic Pressure and Molecular Weight of Serum Albumin in 75 Per Cent\* Glycerol*

Acetate buffer, 0.019 M in HAc, 0.032 M in NaAc; pH 4.9 in aqueous solution; capillary rise, 0.87 cm.; equilibrium time, 15 days; temperature 25°.

<i>C</i> Concentration per 100 cc. solvent	Initial pressure setting	Equilibrium pressure	Density of solution	<i>P</i> Osmotic pressure	$\frac{P}{C}$	<i>M</i> Molecular weight
gm.	cm. solution	cm. solution		cm. H <sub>2</sub> O		gm.
3.60	14.1	11.02	1.190	12.10	3.36	75,200
3.38	9.9	10.45	1.190	11.42	3.38	74,600
Mean.....						74,700

\* Per cent by volume.

state in solution. The phenomenon of denaturation by urea has already been briefly studied by Anson and Mirsky (19), Hopkins (5), Pauli and Weiss (20). While serum albumin denatured by urea is insoluble in water at its isoelectric point, it is very soluble in urea solution. This makes it possible to measure its osmotic pressure in the unionized state in the absence of a Donnan membrane equilibrium.

As in previous work, a 6.66 M urea solution was employed. The measurements at different protein concentrations were made under conditions very nearly identical to previous ones in aqueous solution; namely at the pH of the isoelectric point and in solutions buffered by 0.05 N acetate buffer. The isoelectric point of

denatured serum albumin in 6.66 *M* urea, which will be discussed later, was first determined osmotically and found to be pH 5.8. The urea solutions of denatured protein were prepared from approximately isoelectric and salt-free aqueous solutions of serum albumin by the addition of solid urea. They were kept at least 10 days before the measurements of the osmotic pressure were made. This procedure was followed in order to allow sufficient time for the native albumin to be completely transformed into the denatured form. While the time required for this conversion is at present not accurately known, Hopkins has found that egg albumin at 0° is 90 per cent denatured by urea in 3 days; but serum albumin, it appears from his work, requires a long time.

The relationship of the osmotic pressure to the concentration of serum albumin in urea solution is shown as the upper curve in Fig. 3. The pressure, like that produced in ammonium sulfate solution, increases more than is proportional to increases in the concentration of protein. The relationship describing this deviation from proportionality is different, however, in this non-electrolyte solvent than in the ionic medium, 0.74 *N* (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. When the values of  $\frac{P}{C}$  were plotted against *C* for the solutions containing urea, the points did not fall on a straight line, but a linear relation was obtained by plotting  $\frac{C}{P}$  against *C*, the line sloping down as the protein concentration was increased. This means that the data may be represented by an equation of the type

$$\frac{C}{P} = \frac{1}{K} - B'C \quad (12)$$

Again the relation approaches the form of the ideal osmotic pressure law at infinite dilution, where  $P = RT\frac{C}{M}$  or  $\left(\frac{C}{P}\right)_0 = \frac{M}{RT}$ . The value of the intercept  $\frac{1}{K}$  is therefore  $\frac{M}{RT}$ , or *K* is equal to  $\frac{RT}{M}$  as before. Hence

$$\frac{C}{P} = \frac{M}{RT} - B'C \quad (13)$$

The constant  $B'$  is taken as the slope of the best straight line through the experimental points. The constant  $\frac{M}{RT}$  may be

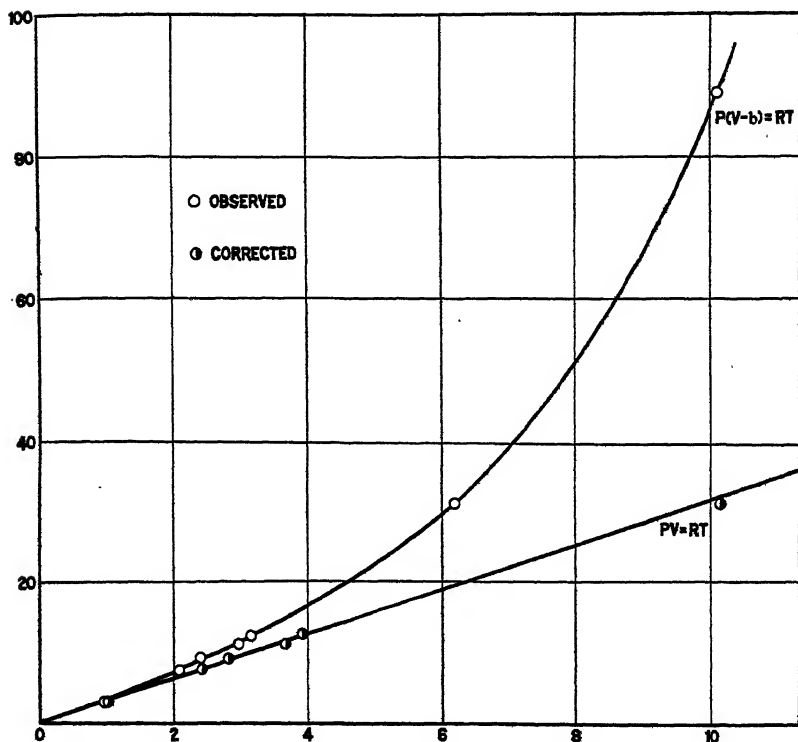


FIG. 3. Osmotic pressure of serum albumin in 6.66 M urea solution at the isoelectric point. Correction of the observed curve for deviation from ideality by Equation 6 yields a linear relationship of pressure to concentration. The ordinate represents the osmotic pressure measured in cm. of water; the abscissa, the concentration in gm. per 100 cc. of solvent.

eliminated by the use of Equation 2,  $P = \frac{RT}{M} C_0$  giving

$$\frac{C_0}{P} = \frac{C}{P} + B'C$$

or

$$C_0 = C + B'PC \quad (14)$$



Equation 14 furnishes a means of calculating  $C_0$  from each point of the data, by using the slope  $B'$  of the line obtained by plotting  $\frac{C}{P}$  against  $C$ . The values so obtained are given in Table IV together with the osmotic coefficients  $g = \frac{C_0}{C}$  calculated from them, and the ratios  $\frac{P}{C_0}$ . The fact that these ratios show no trend is a justification for the use of a single empirical constant  $B'$  in Equation 12. The last column in Table IV gives the values of  $M$  calculated from

TABLE IV

*Molecular Weight of Denatured Serum Albumin from Measurements of Its Osmotic Pressure in Urea Solution*

Acetate buffer; pH 5.8 in 6.66 M urea solution (0.05 M in total acetate); temperature 0°.

Experiment No.	Time of protein in 6.66 M urea	$C$ Concentration per 100 cc. solvent	$P$ Osmotic pressure	$g$ Osmotic coefficient	$C_0^*$ Corrected concentration	$\frac{P}{C_0}$	$M$ Molecular weight
	days	gm.	cm. H <sub>2</sub> O				gm.
45	39	0.97	3.18	1.06	1.03	3.08	75,200
33	14	2.09	7.52	1.15	2.40	3.12	74,300
41	26	2.40	9.32	1.18	2.84	3.28	70,600
84	63	2.97	11.13	1.22	3.63	3.06	75,600
44	35	3.17	12.60	1.25	3.97	3.17	73,100
43	39	6.19	31.0	1.62	10.03	3.09	74,900
51	26	10.11	89.0	2.78	28.11	3.16	73,200
Mean value. ....							73,800

\* Calculated from the equation  $C_0 = C + B'PC$ , where  $B' = 0.02$ .

Equation 2 by the use of these values for  $C_0$ . The fact that their mean is in close agreement with the values obtained from experiments in aqueous buffer solution and in  $(\text{NH}_4)_2\text{SO}_4$  solutions is confirmatory evidence of the validity of the two different methods of correcting the measurements obtained in the different solvents, as well as evidence that the denaturation of serum albumin by urea is not accompanied by a change in molecular weight.

Again it is possible to express the data in terms of an osmotic coefficient  $g = \frac{C_0}{C} = \frac{P}{P_0}$ , as has already been indicated, or to inter-

pret them in terms of solvation of the protein. On the latter basis the true concentration  $C_0$  is that given by Equation 8, and the relation between osmotic pressure and actual concentration would be

$$P = \frac{RT}{M} \frac{100 C}{100 - hC} \quad (15)$$

which follows from Equations 2 and 8, or

$$\frac{C}{P} = \frac{M}{RT} - \frac{MhC}{100 RT} \quad (15, a)$$

The formal identity of Equations 15, *a* and 13 shows that the latter, which was obtained empirically, may be derived on the assumption of a constant degree of solvation for each gm. of protein in the urea solutions. The constant  $B'$  of Equation 12 would then be equal to  $\frac{Mh}{100 RT}$ , where  $h$  is the number of cc. of solvent bound to each gm. of protein. The value of  $h$  calculated from these experiments with serum albumin in urea is 6.3 cc. per gm.

An equation identical with Equation 8 was used by Burk and Greenberg in obtaining corrected concentrations for the calculation of molecular weights from osmotic pressure experiments with casein, edestin, and hemoglobin in urea solutions, and was found by them to apply equally well to the data of others on solutions of hemoglobin in buffer solutions and gelatin in water. Equation 15 seems, therefore, to be a rather general relation; in fact, it is equivalent to the simplest form of van der Waals' equation for gases,

$$P(V - b) = RT$$

which had previously been applied by Sackur (21) and Porter (22) to osmotic pressure measurements with sugar and other crystalloids in aqueous solutions. In connection with the present experiments, it is desired to point out that, while the relation may be deduced on the assumption of a constant degree of solvation, the fitting of the data by such an equation does not prove that solvation is the only cause of the deviations from the ideal law. In fact, it may be preferable simply to describe these deviations by an

osmotic coefficient, which may be related to the concentration by an empirical equation of the form of Equation 16,

$$g = \frac{1}{1 - B' \frac{RT}{M} C} \quad (16)$$

which is derived from Equations 13 and 14.

A few osmotic pressure measurements were also carried out upon serum albumin denatured by heating in aqueous solution. The

TABLE V

*Osmotic Pressure and Molecular Weight of Heat-Coagulated Serum Albumin in Urea Solution*

Solvent 6.66 M urea; 0.05 acetate buffer; pH 5.8; temperature 0°.

A 1 per cent salt-free solution of native serum albumin was heated in a water bath at 70° for  $\frac{1}{2}$  hour. The coagulated protein was separated by centrifuging, washed by suspension in distilled water, and then dissolved in urea-buffer solution.

Experiment No.	$C$ Concentration per 100 cc. solvent	$P$ Osmotic pres- sure	$C_0^*$	$\frac{P}{C_0}$	$M$ Molecular weight
	gm.	cm.H <sub>2</sub> O			gm.
65	0.99	3.29	1.05	3.14	73,600
66	1.72	6.19	1.93	3.20	72,900
Mean .....					72,900

\* Calculated from the relation  $C_0 = C + 0.02 PC$ .

molecular weight of the heat-denatured serum albumin in urea solution was found to be the same as that of the native protein (Table V). As most decomposition reactions are accelerated by increase in temperature, this is further indication of the molecular size stability of this protein in solution. Additional evidence is also shown by the fact that serum albumin denatured by urea or coagulated by heat,<sup>3</sup> may be recovered again as water-soluble crystallizable protein. Thus if the native protein after denaturation by urea<sup>4</sup> or coagulation by heat is dissolved in 6.66 M urea

<sup>3</sup> Cf. Anson and Mirsky (23).

<sup>4</sup> Urea-denatured serum albumin may be obtained as a precipitate from its solution in urea by dialyzing against distilled water at 40-45°.

and dialyzed against distilled water in the cold (2°) until the solution is free from urea, and subsequently electrodialed to remove traces of salt or combined acid or base, a supernatant solution of water-soluble protein is obtained, which yields crystals on half saturation with buffered ammonium sulfate solution.<sup>5</sup> Such

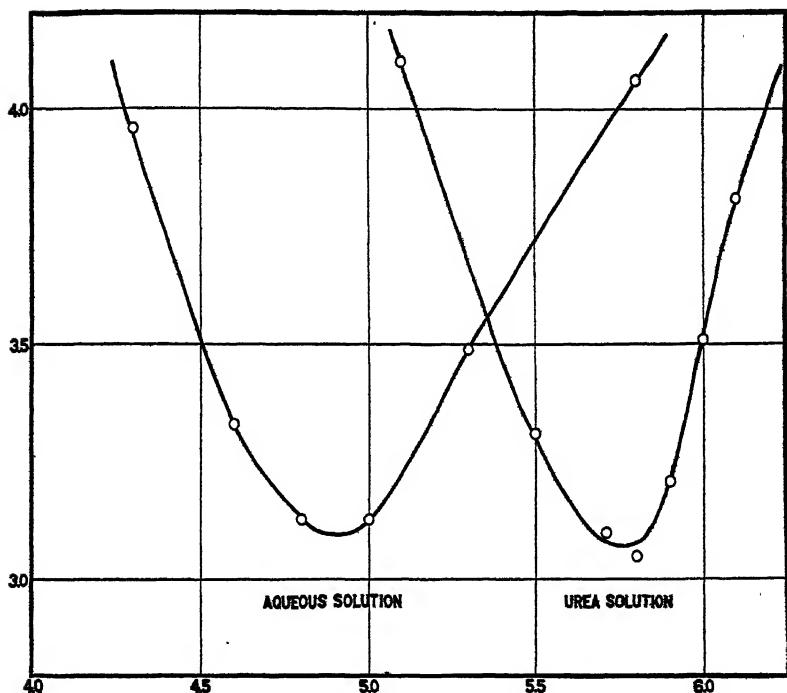


FIG. 4. Influence of pH on the osmotic pressure of serum albumin. (Corrected protein concentration, 3 to 3.5 per cent; buffer concentration, 0.05 M in total acetate; urea concentration, 6.66 M.) The ordinate represents the osmotic pressure per unit of corrected protein concentration; the abscissa gives the pH of the outer solution.

crystals are practically identical, to the eye, with those of native serum albumin. Yields of 25 to 60 per cent of soluble crystallizable serum albumin were obtained from the precipitated, denatured

<sup>5</sup> For carrying out the crystallization, the method of Hopkins as modified by Adair and Robinson (24) was used.

or coagulated protein or from a solution of the protein in 6.66 M urea.

### *Influence of pH*

Measurements of the osmotic pressure of serum albumin in urea solution were carried out in dilute acetate buffer solutions at various hydrogen ion activities in order to locate the reaction of minimum osmotic pressure in this solvent. For the purpose of comparison, similar measurements were also made upon our preparation of serum albumin in aqueous solution under approximately the same conditions. The results are graphically shown in Fig. 4, in which it is seen that in urea solution serum albumin exhibits a minimum in osmotic pressure at pH 5.8, while in aqueous solution, at pH 4.85 to 4.9. The latter figure is in fair agreement with the isoelectric point of this protein as determined by other investigators employing methods of cataphoresis (Table VI). Fig. 4 also shows that the change in osmotic pressure with change in pH, as manifested by the steepness of the osmotic pressure-pH curve on either side of the isoelectric point, is just as great, if not more so, for denatured serum albumin, as it is for native serum albumin. This relationship has a certain bearing on the question of whether the denaturation of a protein involves a loss of its free carboxyl and amino groups, as has been assumed by some in certain theories of denaturation. As shown by Loeb (25) in his studies upon the application of Donnan's theory of membrane equilibrium to protein solutions, increases in osmotic pressure produced by changes in pH on either side of, and close to, the isoelectric point are dependent upon increases in ionization of the protein if conditions of protein and salt concentration are constant. In the measurements graphically represented in Fig. 4, these conditions were approximately maintained.<sup>6</sup>

Since the minimum in the curve representing denatured serum albumin is not any less sharp than that representing the native protein, it follows that denatured serum albumin, when dissolved, possesses an ability to ionize in the region of the isoelectric point,

<sup>6</sup> In the aqueous solutions over the pH range studied the mean concentration of the salt, sodium acetate, was 0.038 M; while in the urea solutions, 0.03 M. The protein concentrations in the former solutions were about 3.5 per cent and the corrected concentration in the latter about 3 per cent.

at least equally as great as native serum albumin. It is, therefore, highly probable that the denaturation of albumin is not accompanied by a decrease in acid- and base-combining power, as would result if this change involved the union of free carboxyl and amino groups to form anhydrides.

In adding urea to an aqueous solution of serum albumin, changes both in the nature of the solvent and the character of the protein are introduced. The more alkaline isoelectric point (point of minimum osmotic pressure) of serum albumin in urea solution may, therefore, be due to some factor other than denaturation. It seemed important to ascertain by supplementary means which

TABLE VI  
*Measurements of the Isoelectric Point of Serum Albumin in Aqueous Solution*

Investigator	Year	Isoelectric pH	Buffer concentration	Method
Michaelis and Davidsohn (26).....	1911	4.70	0.02-0.1 M in Na acetate	Zero migration by cataphoresis
Ito and Pauli (27).....	1929	4.99	0.005 M in total acetate	" "
Tiselius (28).....	1930	4.88	0.002 M in Na acetate	Rates of migration by moving boundary
Ito (29).....	1931	4.88	0.04 M in total acetate	Zero migration by cataphoresis
		4.85-4.90	0.05 M in total acetate	Minimum osmotic pressure

factor, the environmental (the medium effect of urea itself) or the constitutional (the denaturation change or its accompaniments), is responsible for providing this observed change in electrochemical properties. For this purpose an estimation of the isoelectric point of urea-denatured serum albumin was made by observing its flocculation (maximum insolubility) in aqueous buffer solutions at various pH values. Urea-denatured serum albumin was obtained as a precipitate from its solution in 6.66 M urea by dialyzing and electro-dialyzing such a solution against distilled water. The observations upon its flocculation are recorded in Table VII. For comparison the similar observations of Michaelis and Davidsohn (26) and the nephelometric measurements of Teorell (30)

upon the flocculation of serum albumin denatured by heat are also given in Table VII. It is seen from Table VII that serum albumin, after denaturation by urea, exhibits in aqueous solution a mid-

TABLE VII  
*Flocculation of Denatured and Coagulated Serum Albumin in Aqueous Acetate Buffer Solutions*

+ indicates presence of flocculated protein. Figures represent relative turbidity as measured by a nephelometer.

Denaturing or coagulating agent.....	6.66 M urea	Heat	Heat	First by heat, secondly by urea
Acetate buffer concentration.....	0.05 M	0.005 M	0.2 M	0.05 M
Initial protein concentration, per cent.....	>0.05	3	0.09	>0.05
Observer.....		Michaelis	Teorell	
pH				
4.3.....	Clear		0.17	Same as in 1st column
4.5.....	Slight ppt.		1.3	
4.7.....	Ppt.		1.4	
4.9.....	"		18.5	
5.1.....	"	+	19.1	
5.3.....	Opalescent	++	19.2	
5.4.....	Clear	+++	19.2	
5.5.....	"	++	19.2	
5.7.....	"	+	19.2	
5.9.....	"		19.3	
6.1.....	"		0.42	
6.3.....	"			
pH of flocculation mid-point.....	4.80-4.90	5.4	5.4	

\* pH of this solution, 5.2.

point in its zone of maximum insolubility at a pH value approximately identical, within the limits of accuracy of the method employed, with the isoelectric point of native serum albumin,

indicating the absence of any change in isoelectric point on denaturation of this protein.<sup>7</sup> The more alkaline point of minimum osmotic pressure exhibited by serum albumin in urea solution may therefore be attributed entirely to the medium effect of the urea itself,<sup>8</sup> which presumably causes a change in the dissociation of the ionizing groups originally present in the native molecule; for upon removal of the urea this change in dissociation is apparently reversed even though the denaturation produced by urea persists.

A comparison of the flocculation of serum albumin denatured by urea with that coagulated by heat (Table VII) shows that the action of these two denaturing agents is different. While both agents give rise to a product insoluble in water, their flocculation maxima lie at different pH values. Heat-coagulated serum albumin is apparently isoelectric at pH 5.4 since Michaelis and Davidsohn confirmed their value obtained from flocculation experiments by cataphoretic measurements. Heat coagulation appears to be a more complex change, involving in addition to simple denaturation,<sup>9</sup> further alteration in the molecule; for if serum albumin which has been denatured by urea is heated in dilute aqueous suspension for a few minutes it undergoes modification in that its zone of insolubility in acetate buffer solutions extends into a more alkaline pH region.

The writer wishes to express his thanks to Professor E. J. Cohn for his interest in this work during its progress in 1929-31.

#### SUMMARY AND CONCLUSIONS

1. From measurements of the osmotic pressure upon crystalline horse serum albumin in aqueous buffer solution at the isoelectric point, under which conditions the pressure conforms to the ideal law, the mean molecular weight was found to be 74,600. Other

<sup>7</sup> This result is in agreement with that of Hendrix and Wilson (31) who report that egg albumin denatured by 0.1 N HCl and flocculated at its isoelectric point, shows the same acid- and base-combining capacity as undenatured egg albumin.

<sup>8</sup> Cf. Burk and Greenberg (6) regarding the effect of urea on the dissociation of simple buffers.

<sup>9</sup> The reader is referred to the paper of Wu (32) for rigid definitions of the terms denaturation and coagulation.



figures reported in the literature are: 72,000 (Adair and Robinson), 67,500 (Svedberg and Sjögren), 45,000 (Sörensen).

2. Measurements of the osmotic pressure in ammonium sulfate solution upon albumin once crystallized confirmed those of Sörensen upon protein crystallized several times, which were made at high protein concentration, and showed that in moderately concentrated salt solution the pressure does not conform to the ideal law, but increases more than proportional to increases in protein concentration. When corrected for this deviation from the ideal law, a value of 76,000 for the molecular weight is obtained both from our measurements and those of Sörensen. The view-point that serum albumin is decomposed into products of low molecular weight by treatment with ammonium sulfate is, therefore, not supported in these experiments.

3. Serum albumin, denatured by urea or by heat, was found to have the same molecular weight in urea solution (73,000) as native serum albumin in aqueous solution.

4. The osmotic pressure of dilute solutions of serum albumin in 75 per cent glycerol solution was found to be practically the same as that in aqueous solution.

5. The isoelectric point of serum albumin denatured by urea was estimated from its maximum flocculation in aqueous buffer solutions to be at pH 4.8 to 4.9, showing no change of isoelectric point on denaturation. Urea-denatured serum albumin in solution in 6.66 M urea showed a minimum in osmotic pressure at pH 5.8. The more alkaline isoelectric point of the protein in urea solution is, therefore, probably due entirely to the medium effect of urea.

6. It is concluded that serum albumin is a protein of fairly stable molecular weight.

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## ON THE EFFECT OF ARSENATE ON BLOOD GLYCOLYSIS

### A CORRECTION

By A. E. BRAUNSTEIN

(From the Biochemical Institute of the Peoples Commissariat of Public Health, Moscow, U. S. S. R.)

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In their paper on the effect of arsenate on blood glycolysis Morgulis and Pinto (1932) discuss among other data those of Engelhardt and Braunstein (1930) and of Braunstein (1931). To our experimental technique and theoretical arguments some objections are raised by Morgulis and Pinto, indicating that they overlooked several points stated in our work and in consequence have misinterpreted it. Some explanatory remarks appear therefore necessary.

1. Morgulis and Pinto write: "Braunstein using the Fiske-Subbarow method for the phosphate determination depends upon rapid reading of the colors to avoid errors due to the presence of arsenate, the extra color development from arsenate presumably being a slower process." This procedure of quantitative determination is termed "uncertain and indeed . . . very uncritical." These authors fail to notice the fact mentioned in both the articles by Engelhardt and by Braunstein that *a modification of the Fiske-Subbarow method* was used, as described by Braunstein (1928). Further it is explicitly stated in both of our papers that *a correction* for arsenate was applied to the phosphate determinations.

The method employed by us for the estimation of inorganic phosphate is essentially an adaptation of Lohmann and Jendrasik's (1926) modification of the Fiske-Subbarow method for use with smaller amounts of material (the final volume of the reaction mixture being 2.5 to 5 cc.); further improvements are introduced concerning the technique of the total and acid-soluble phosphorus determinations. The procedure of Lohmann and Jendrasik involves the use of a molybdate solution of higher acidity than does

the original method of Fiske and Subbarow and the warming of the material on a water bath at 37° for 4 or 5 minutes to complete the color development. Immediately on removal from the water bath and cooling, a solution of arsenate treated according to this technique displays a very slight blue coloration; up to  $M/600$  to  $M/300$  solutions this color is too faint for colorimetry; on standing at room temperature the color gradually darkens, and in the course of several hours grows deeper by far than the color developed by

TABLE I

*Colorimetric Determination of  $P_2O_5$  in Standard Solutions of Potassium Phosphate in Presence of Arsenate*

(Illustrating the correction to be taken into account and the rôle of the time factor.)

Test solutions plus reagents were warmed to 37° for 4 minutes; then cooled under the tap. Room temperature, 14°.

Time after removal from water bath	$M/600$ $Na_2HAsO_4$	$M/600$ $KH_2PO_4$	$M/600$ $KH_2PO_4$ + $M/600$ $Na_2HAsO_4$	0.002 M $KH_2PO_4$	0.002 M $KH_2PO_4$ + $M/600$ $Na_2HAsO_4$
	Colorimeter readings, mg. per cc. $P_2O_5$				
min.					
1	0	0.114	0.114	0.141	0.142
2	0*		0.117		0.144
4	0.004		0.118		0.145
6	0.004*		0.120		0.146
8	0.004*				0.146
10			0.122		0.148
12	0.008*				0.150
15			0.126		0.152
20	0.012*	0.114	0.130	0.142	0.155

\* Color too pale for exact colorimetric estimation.

an equivalent amount of phosphate. The color developed in mixtures of phosphate and arsenate is additive for any given moment of time. The rate of color development in  $M/600$  arsenate as well as in mixtures with phosphate is shown by the results of an experiment performed in order to work out a correction graph for  $M/600$  arsenate in phosphate determinations (Table I).

It may be seen that the correction is less than 0.005 mg. per cc.

of  $P_2O_5$ , provided the colorimetric reading be taken within 5 minutes after removal from the water bath, the moment of the reading, of course, being exactly noted. As the concentration of inorganic phosphate in our experiments usually has the order of magnitude of 0.060 to 0.200 mg. per cc., or 0.001 M to M/300, the correction reaches 10 per cent at most.

As opposed to this, Morgulis and Pinto use the Kuttner and Cohen (1927) method of phosphate determination, likewise deducting a correction for arsenate. They state that "the color produced by As is more than 3 times as intense as that produced by an equivalent amount of P." This would mean that the colorimeter reading obtained with a M/600 solution of arsenate corresponds to about 0.500 mg. per cc. of  $P_2O_5$ , with 0.002 M arsenate (a concentration used in some experiments by Morgulis and Pinto) to more than 1.500 mg. per cc. of  $P_2O_5$ . The correction, therefore, would be 100 to 300 times as large as in our method; in fact, it would amount to three- to tenfold the actual content of inorganic phosphate to be expected in blood samples.

2. Our hypothesis as to the possibility of hexosearsenate formation is discarded by Morgulis and Pinto on account of their failure to observe by means of their colorimetric method a diminution in the total color evolution that would correspond to an esterification of arsenate equivalent in amount to the phosphate liberated.

We, of course, never held the opinion and never asserted that stable hexosearsenates would be synthesized and accumulated by the glycolyzing blood cells. Only an intermediate formation of a highly unstable compound followed under conditions approximating the physiological state by immediate breakdown to inorganic arsenate and products of hexose disintegration is to be expected.<sup>1</sup> Neither are the phosphorus compounds that are disintegrated in non-glycolyzing blood and synthesized during glycolysis hexosephosphoric esters. It was shown by our experiments (Braunstein, 1931) that they belong partly to the pyrophosphate, partly to the diphosphoglycerate fraction. Their

<sup>1</sup> This assumption is corroborated by experiments of Braunstein and Levitov (Braunstein, A., and Levitov, M., *Naturwissenschaften*, June (1932); *Biochem. Z.*, in press) on the esterification of inorganic arsenate by fermenting yeast preparations.

formation apparently is connected or coupled in some way or other with the intermediate phosphorylation of the blood sugar during glycolysis. According to our view, the action of arsenate depends on its impeding the esterification of the hexose with phosphate by "competitive inhibition" and thus preventing the resynthesis of the organic phosphorus compounds mentioned above, whereas their disintegration by the phosphatases of the blood goes on unimpaired.

3. Our statement that arsenate does not produce any acceleration of the breakdown of phosphorus compounds in non-glycolyzing blood, where there is no overlapping of the hydrolytic process by resynthesis, is misunderstood by Morgulis and Pinto as indicating that we negate the possibility of the phosphatase and the glycolysis enzyme systems being affected independently. It is evident from our publications that we always maintained the opposite view. The fact observed by Morgulis and Pinto that arsenate always promotes the liberation of inorganic phosphate regardless of whether this is accompanied by an acceleration of glycolysis or a partial inhibition (as in dog blood), is due to the precluding of resynthesis and corroborates (as does the similar effect of  $\text{CaCl}_2$  in the experiments of Roche and Roche (1929)) the view of Engelhardt and Braunstein that the reesterification of phosphate is a phenomenon connected with glycolysis, but not a necessary condition of the latter.

4. The explosive increase of the inorganic phosphate during hemolysis alluded to by Morgulis and Pinto as "the maximum liberation of phosphate," is due to the breakdown of pyrophosphate, first investigated by Engelhardt (1930). It is followed by a comparatively slow disintegration of other phosphorus compounds (chiefly diphosphoglycerate). To this latter process our statement relates that arsenate does not affect the hydrolysis of phosphate esters in hemolyzed blood.

5. Morgulis and Pinto assign to Macfarlane the assertion that arsenate stimulates phosphatase activity. In fact Macfarlane (1930) observed that arsenate does not influence the liberation of inorganic phosphate from hexosediphosphate by cozymase-free yeast preparations. Only when a simultaneous fermentation of the hexosephosphate is taking place, there is an increase of the rate of dephosphorylation by arsenate. Macfarlane concludes

that the action of arsenate is not due to a direct effect upon the hydrolysis of hexosediphosphate, but is a phenomenon of complex character, being connected somehow with the action of cozymase (and possibly with some further factor contained in the coenzyme fraction). In a recent survey of the biochemistry of alcoholic fermentation, Harden cited in Nord-Weidenhagen (1932) sustains the view, based mainly on the data of Macfarlane, that the "arsenate effect" depends on the activation of a special enzymic mechanism performing the direct fermentation of hexosediphosphate in the presence of cozymase, rather than on the stimulation of the hydrolytic enzyme phosphatase.

Since hemolyzed blood contains cozymase and is capable of glycolyzing added hexosediphosphate to an appreciable extent, the stimulation by arsenate of phosphate liberation from hexosephosphate in hemolyzed blood, observed by Morgulis and Pinto,<sup>2</sup> is by no means a decisive proof that arsenate acts through direct stimulation of hexosephosphatase. The objections put forward by Macfarlane and by us against such an interpretation of the acceleration of hexosephosphate breakdown by arsenate apply to these experiments as well. With the glycolytic enzyme system of erythrocytes totally hemolyzed at low temperature, Meyerhof (1932) observed no stimulating effect of arsenate on the breakdown of hexosediphosphate.

To conclude with, it must be stated that the greater increase in inorganic phosphate and the slower rate of glycolysis observed by the authors in physiological NaCl solution than in Locke's solution is readily explained by the damaging action of dilution with normal saline on the glycolytic system of blood cells, described by Engelhardt and Braunstein (1930). Figs. 4 and 5 of the paper of Morgulis and Pinto (1932) show the additive results produced by the specific action of arsenate and by the damaging of the blood cells with normal saline.

The observation of Morgulis and Pinto, in itself interesting, that dog blood responds to arsenate by a diminished glycolysis, is

<sup>2</sup> The method of hemolysis used by these authors is not altogether satisfactory; the addition of an equal volume of distilled water to blood cells produces very incomplete cytolysis. Either considerably larger amounts of distilled water or ether-saturated water should be employed in order to insure total laking of the blood.



perhaps connected with the well known fact of its relatively weak glycolytic activity (Engelhardt and Ljubimowa, 1930) and with the rapid decline of the rate of the glycolytic process, illustrated by Fig. 3 in the paper of Morgulis and Pinto. This seems to indicate that the glycolytic system of the dog erythrocytes is rather unstable. It might be that it is more susceptible to the toxic influence of arsenate than that of rabbit erythrocytes which also are damaged by higher concentration of arsenate (Braunstein, 1931). The observations of Meyerhof (1921, 1927) on the arsenate effect in muscle and muscle preparations offer another instance that the action of arsenate, especially with regard to the concentrations producing stimulation, depends to a considerable extent upon the nature and condition of the individual glycolytic system. The toxic effect of course must have been especially marked in those experiments of Morgulis and Pinto where the glycolytic system had been damaged by normal saline or by prolonged incubation (4 hours). In fact there is no inhibition of glycolysis by arsenate apparent in dog blood cells incubated for 2 hours, to judge from Fig. 2 of Morgulis and Pinto; there seems rather to be a slight stimulation.

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# STUDIES ON BLOOD GLYCOLYSIS

## I. EFFECT OF ARSENATE

### A REPLY

By SERGIUS MORGULIS AND SHERMAN PINTO

(From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha)

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In his polemical note Braunstein (1) discusses at some length, and in a very satisfactory manner, how he has made the phosphorus determinations in the presence of arsenate. Had this information been given in his paper there would have been no occasion for misunderstanding his procedure. But in his paper he said: "*Bei der Phosphorsäure Bestimmung in Gegenwart von Arsenat ist stets darauf zu achten, dass die Proben sofort nach Entnahme aus dem Wasserbad kolorimetriert werden, da sie sonst stark nachdunkeln.*" Of course, we never doubted the reliability of his results, but in the absence of any other information we interpreted the quoted remark to mean that the separation of the P from the As was attempted on the basis of the different rates of color development. Under the circumstances, this was a reasonable inference and justified our casual comment that this is hardly a critical procedure for quantitative estimations.

Working with the Kuttner method (2) for the determination of phosphates by which As develops an intense color, we felt especially justified in making such an observation, but we found out that by the Lohmann-Jendrassik modification of the Fiske-Subbarow method a much weaker color develops with arsenate although the final acidity is the same in both. Braunstein refers to his own special *modification* of the Fiske-Subbarow procedure, but we are utterly at a loss to find out what this modification is. We have gone through carefully his original description (3) of the presumed modification (published in Russian) but find that the procedure as it is given there is identical with that of Lohmann and Jendrassik (4).

The fact that As gives a more intense color with the Kuttner than with the Fiske-Subbarow phosphate procedure has turned out a rather fortunate accident which has helped to clarify the problem of hexosearsenate ester formation. To be sure, we did not employ the Kuttner method intentionally with this end in view, but, as we pointed out in our paper, had such esterification with arsenate occurred it would have caused a decrease in color because of the difference in color developed by P and As, while just the reverse was actually the case in all our experiments. If we understand Braunstein correctly, his present belief seems to be that arsenate merely prevents esterification of hexose with phosphate while dephosphorylation is unimpaired by the arsenate, whereas in his paper he assumed that the accelerating effect of As on glycolysis was due to the formation of a more easily glycolyzable hexose-arsenate ester. To be sure, this was suggested merely as a hypothesis and we feel that our happy, though not deliberately planned, choice of the Kuttner method has helped to dispose of this hypothesis. In arguing about the various points we have raised in our paper (5), Braunstein fails to consider the one really important thing; namely, that arsenate may either accelerate or inhibit the glycolytic process but that it invariably increases the hydrolysis of the phosphates. The studies which we made on the blood phosphatase were carried out exactly in accordance with Roche's technique and show unmistakably a direct relationship between the arsenate concentration and the acceleration of the phosphate hydrolysis.

We regret very much that in our reference to Macfarlane's findings on the effect of arsenate on the phosphatase we have lapsed into a grievous error. Though unintentionally, we have misstated her position and we are thankful to Braunstein for calling our attention to this, so that we can correct the error. We should have said that Macfarlane (6) found that arsenate stimulates phosphatase activity of yeast preparations *only in the presence of some activator*. On the other hand, Braunstein also errs when he states that Macfarlane found the hydrolysis of hexosediphosphate to be accelerated by arsenate *only in the presence of cozymase*. That arsenate does not affect equally phosphatases of different origin has been known since Neuberg and Leibowitz (7) showed that kidney phosphatase is unaffected by arsenate, that taka-phosphatase is

only slightly affected, but yeast phosphatase activity is greatly increased. Macfarlane's interesting studies may perhaps supply the clue to the factor essential for the accelerating action of arsenate, but when Braunstein unqualifiedly identifies this as the cozymase he goes much further than Miss Macfarlane herself is willing to admit. Speaking of her interesting experiments, she says very plainly that "this points to some other factor besides co-enzyme being concerned in the mechanism" of phosphatase hydrolysis. In another connection she expresses herself even more definitely: "While no definite conclusion that the accelerating action of arsenate on the liberation of phosphate is invariably accompanied by the fermentation of hexosediphosphate can yet be reached, the evidence here offered indicates that the phenomenon is certainly more complex than has hitherto been realized." On this score we are in entire agreement with Miss Macfarlane. We are dealing with a complex phenomenon in the matter of the As effect, as our own experiments of its different behavior in different glycolyzing systems manifest, and we believe that if any satisfactory solution of this problem will be attained it will be through experimentation rather than argumentation.

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## THE EFFECT OF THE CALCIUM-PHOSPHORUS RELATIONSHIP ON GROWTH, CALCIFICATION, AND BLOOD COMPOSITION OF THE RAT\*

By R. M. BETHKE, C. H. KICK, AND WILLARD WILDER

(From the Department of Animal Industry, Ohio Agricultural Experiment Station, Wooster)

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Numerous investigators have shown that the three dietary components, calcium, phosphorus, and vitamin D, are especially concerned in normal bone formation. McCollum and associates (1) and Sherman and Pappenheimer (2) showed independently that the quantitative relation between the calcium and phosphorus in the food supply is, within certain limits of concentration, of considerable significance in determining whether the rat will develop bones which are normal or pathological. Likewise, Goldblatt (3) reported that the change in the Ca:P ratio from 1:0.88 to 1:0.20, in a purified synthetic diet low in phosphorus (0.22 per cent) and the fat-soluble organic factor, through the addition of calcium carbonate had the effect of changing it from a non-rickets-producing to a rickets-producing diet.

Karelitz and Shohl (4) reported that rats made rachitic with a high calcium-low phosphorus ration, devoid of vitamin D, were cured by the addition of monobasic sodium phosphate to the ration.

Bethke, Steenbock, and Nelson (5) pointed out that with rats on low calcium rations the quantity of cod liver oil necessary to furnish the antirachitic factor in sufficient amounts varied inversely with the calcium content of the ration. The same conclusion was reached in the case of the rat on moderately high phosphorus rations. Haag and Palmer (6) also showed the importance of a more or less balanced condition of calcium, phosphorus, and magnesium salts in the ration for good growth and mineral retention in

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the rat. The same investigators call attention to the importance of the vitamin-mineral relationship by stating, "The response of various groups of animals [rats] to certain vitamin levels points to even more important vitamin-mineral interrelationships than have heretofore been recognized."

Medes (7), in studies of the effect of varying mineral ratios on the mineral composition of the rat, reported that the normal composition of the rat with respect to calcium and phosphorus (mg. per gm. of weight) cannot be altered by increasing the amount of phosphorus in the diet above that needed for normal growth. She also reported that altering the P:Ca ratio from 0.73 to 1.87 did not have any effect as long as both elements were present in adequate amounts and the calcium was not present in massive doses. The same investigator reported that rats which received diets containing 0.12 per cent phosphorus and 0.37 per cent calcium developed severe rickets. This is in accordance with the report of Pappenheimer, McCann, and Zucker (8) who state that when the phosphorus of the diet is reduced to 0.135 per cent, rickets begins to appear and is active when phosphorus is present only to the amount of 0.11 per cent.

The practical significance of adequate amounts of calcium and phosphorus in the food supply is imperative. It is also recognized that a marked disproportion in the calcium and phosphorus of the diet, in absence of vitamin D, seriously interferes with calcification and constitutes one of the dietary essentials for the production of experimental rickets in the rat. Information, however, on the interrelation of these elements at different levels or concentrations, in the absence or presence of vitamin D, is meager. The present report deals principally with some of these fundamental questions with respect to bone formation in the rat. The data are, in part, from a series of investigations started several years ago to study the importance of the calcium-phosphorus relationship in the nutrition of the rat, pig, and chick.

#### EXPERIMENTAL

At the start of the series of investigations it was thought advisable, for experimental reasons, to use comparable rations as much as possible. To this end, we had in mind a ration which could be relied upon to produce incipient rickets in the rat in 3 to 5 weeks

and still, in the case of the pig, constitute a comparatively practical ration. After a great deal of preliminary investigation, a ration of 79.5 parts of yellow corn, 20 of soy bean oil meal, and 0.5 of sodium chloride, was chosen. This ration, when supplemented with 3 parts of calcium carbonate, produced severe rickets in rats of our breeding in 3 to 4 weeks.

In all of the experiments rats reared on our stock ration were used. They were started on experiment when 23 to 25 days of age and weighing from 45 to 60 gm. All groups were confined in wire cages provided with false screen bottoms and cared for in a room devoid of sunlight. With few exceptions, the sex distribution was equal in all groups in a particular experiment.

For calcification studies, the femurs, ulnæ, and radii were removed from each rat. The femurs were freed of adhering tissue, dried, and then extracted for 18 to 24 hours with hot alcohol and subsequently with ether for a similar period. The dried extracted bones were then ashed in an electric muffle furnace. The per cent ash is calculated on a moisture and fat-free basis. The ulnæ and radii were fixed in 4 per cent formalin and examined for degree of calcification by the silver nitrate procedure.

The first experiment consisted of five groups of rats, six in each group. They received the yellow corn-soy bean meal ration, supplemented with varying amounts of calcium and phosphorus, as shown in Table I. At 5 weeks, two animals from each group, except Group 2, were killed for bone analysis. The remaining animals were continued on experiment for another 12 weeks, when they were killed for calcification studies. The results are presented in Table I.

The group that received the unsupplemented basal ration, exhibited signs of a marked mineral deficiency, in the form of a rough hair coat and stiffened, staggering gait, at an early date. The addition of 3 parts of calcium carbonate to the basal mixture produced severe rickets, as attested by the low ash content (21.2 per cent) and gross examination of the ulnæ and radii. These animals were in an extremely rachitic condition and probably would not have survived longer. When the basal mixture was supplemented with 3 parts of secondary sodium phosphate and 3 parts of calcium carbonate, the ash content of the bones and their histological picture were normal. Similar results were ob-



tained when the ration was fortified with calcium and phosphorus in the form of ground limestone and bone meal. The beneficial effects of ultra-violet irradiation on growth and bone formation are revealed in Group 3 (Table I). It is interesting to note that the growth of the irradiated group did not equal that of the groups that received additional phosphorus in the form of bone meal or sodium phosphate, even though the latter groups received little if any vitamin D.

TABLE I

*Effect of Varying Calcium and Phosphorus Intake on Growth and Ash Content of Femurs*

Group No.	Additions to basal ration	Ca in ration	P in ration	Ca:P ratio	Wks. on ration	No. of animals	Average gain in weight	Average ash in femurs
		per cent	per cent				gms.	per cent
1	None	0.06	0.33	0.18	5 17	2 4	38 66	37.1 37.0
2	3 calcium carbonate	1.16	0.32	3.62	5	6	36	21.2
3*,†	3 " " (irradiated)	1.16	0.32	3.62	5 17	2 4	56 134	45.5 62.9
4*	3 calcium carbonate, 3 disodium phosphate	1.14	0.62	1.84	5 17	2 4	75 173	49.4 65.1
5†	1 ground limestone, 1 bone meal	0.62	0.44	1.41	5 17	2 4	88 156	45.1 62.6

\* Group irradiated 10 minutes daily, six times per week, with quartz mercury vapor lamp at a distance of 30 inches.

† One female in group had young but they were not nursed.

The data, in general, are in agreement with previously reported investigations—that vitamin D and the quantitative relation between the calcium and phosphorus in the ration are important factors in calcification. The next logical step appeared to be to study the influence of small progressive increments in calcium and phosphorus on growth and bone formation. For this purpose the aforementioned basal ration of yellow corn, soy bean oil meal, and salt was supplemented with increasing amounts of calcium carbonate alone, and calcium carbonate and secondary sodium phosphate and fed to rats in groups of four each. All groups, unless otherwise indicated, were on experiment for 12 weeks, when they were sacrificed for bone analysis.

The data recorded in Table II show that without the further addition of phosphorus to the basal mixture, the ash content of the femurs was progressively reduced when more than 1 per cent of calcium carbonate was added to the ration. When the per cent phosphorus in the ration was increased, with corresponding in-

TABLE II

*Effect of Varying Calcium and Phosphorus Intake on Growth of Rats and Ash Content of Their Femurs (No Vitamin D Added)*

Mineral additions to ration		Ca in ration	P in ration	Ca:P ratio	Average gain in weight	Average ash in femurs	Condition of animal
CaCO <sub>3</sub>	NaHPO <sub>4</sub>						
parts	parts	per cent	per cent		gm.	per cent	
		0.06	0.33	0.18	44	32.4	Stiff, poor
0.5		0.24	0.33	0.73	108	51.1	Good
1.0		0.42	0.33	1.27	103	51.4	"
2.0		0.79	0.33	2.39	48	28.5*	Rachitic
3.0		1.16	0.32	3.62	39	23.5*	Very rachitic
	1.0	0.06	0.43	0.14	48	33.3	Stiff, poor
0.5	1.0	0.24	0.43	0.56	86	45.3	Some stiffness, fair
1.0	1.0	0.42	0.43	1.00	104	60.6	Good
2.0	1.0	0.78	0.42	1.86	116	52.2	" †
3.0	1.0	1.15	0.42	2.74	71	29.0	Rachitic
	2.0	0.06	0.53	0.11	45	31.4	Stiff, very poor†
0.5	2.0	0.24	0.53	0.45	88	41.5	" but fair
1.0	2.0	0.41	0.52	0.79	112	55.6	Good§
2.0	2.0	0.78	0.52	1.50	90	59.2	Good
3.0	2.0	1.14	0.51	2.23	89	57.1	"

\* Killed at 5 weeks.

† One animal died in 2nd week.

‡ One animal died in 12th week.

§ Two females had 3 litters of young, not nursed.

creased amounts of calcium, the animals tolerated a greater per cent of calcium, as reflected in increased growth and bone ash—again showing that the proportion of calcium to phosphorus in the ration is of great significance in calcification. The addition of 0.2 per cent of phosphorus, in the form of secondary sodium phosphate, to the basal ration, supplemented with 0.5, 1.0, 2.0, and

3.0 per cent of calcium carbonate, further reflected the importance of the inorganic relationship in bone formation. A ration which normally produced incipient rickets in 3 to 4 weeks was made non-rachitogenic by adding 2 parts of secondary sodium phosphate. It is also evident that the concentration or per cent of phosphorus in the ration had some effect on bone ash. A Ca:P ratio of 2.39 at a phosphorus level of 0.33 per cent caused rickets, whereas a 2.23 ratio with the phosphorus increased to 0.51 per cent made for a nearly normal ash content.

Other experiments with the same basal ration confirmed the results of the previous experiments—that the proportion of calcium to phosphorus in the ration of the rat is of great significance in bone formation. Evidence was also obtained which indicated that the amount of vitamin D required for normal calcification was dependent upon the Ca:P ratio of the ration. The addition of either tertiary calcium phosphate or calcium carbonate and secondary sodium phosphate to the basal mixture to establish a Ca:P ratio between 1.00 and 2.00 made for a practically normal ash value which was but slightly increased by the addition of vitamin D.

Although the results showed rather conclusively the importance of the Ca:P ratio in the nutrition of the rat, it still remained to be determined whether the concentration or level of these two elements at a particular proportion also exerted an effect on calcification. It being assumed that there exists an optimum calcium-phosphorus relationship at a given level or concentration, are the vitamin D requirements of the particular animal at a minimum at this relationship? Or, to state it in another way, is it possible to reduce the antirachitic requirements of an animal to a minimum by properly adjusting the calcium and phosphorus content of its ration?

With these questions in mind, a fourth experiment, which involved the feeding of the yellow corn-soy bean oil meal ration supplemented with such amounts of calcium carbonate, secondary sodium phosphate, and tertiary calcium phosphate, to obtain eight different Ca:P ratios at three different levels or concentrations, was started. The general plan of the experiment, with the per cents calcium and phosphorus in the different rations, are shown in Table III. Each ration was fed to two groups of four

rats each. Irradiated ergosterol dissolved in linseed oil was added to the ration of one group in each pair, while the other group served as a negative control. The oil solution was added on a 0.5 per cent basis. 1 cc. of the solution was equivalent to approximately 200 Steenbock units of vitamin D. All groups in one particular series were started at the same time. The foodstuffs and salts used in the low and the medium concentration series were from the same stock. In the high series a new batch of yellow corn, soy bean oil meal, calcium carbonate, and secondary sodium phosphate were used.

TABLE III

*Plan of Experiment, Showing Per Cent of Calcium and Phosphorus in Rations*

Ca:P ratio	Low concentration*		Medium concentration†		High concentration‡	
	Calcium	Phosphorus	Calcium	Phosphorus	Calcium	Phosphorus
5.00	1.79	0.36	2.64	0.53	3.24	0.65
4.00	1.44	0.36	2.14	0.53	2.64	0.66
3.00	1.09	0.36	1.63	0.54	2.01	0.67
2.00	0.74	0.37	1.10	0.55	1.36	0.68
1.00	0.37	0.37	0.56	0.56	0.69	0.69
0.50	0.37	0.73	0.55	1.09	0.67	1.34
0.33	0.36	1.08	0.53	1.60	0.65	1.95
0.25	0.35	1.42	0.52	2.08	0.63	2.53

\* Basal ration plus varying amounts of calcium carbonate and secondary sodium phosphate.

† Basal ration with 1 per cent calcium phosphate plus varying amounts of calcium carbonate and secondary sodium phosphate.

‡ Basal ration with 2 per cent calcium phosphate plus varying amounts of calcium carbonate and secondary sodium phosphate.

The groups were continued on their respective rations for 5 weeks, when they were sacrificed for blood and bone analyses. To obtain blood samples, the rats were anesthetized with ether and bled from the carotid artery. The blood from each group was pooled and allowed to stand overnight in the refrigerator. The following morning the serum was pipetted off, centrifuged to remove contaminating corpuscles, and immediately analyzed for calcium (9) and inorganic phosphorus (10). The femurs, ulnæ, and radii were treated as previously described.

The inorganic phosphorus content of the blood serum, presented in Table IV, was greatly reduced in the groups which did not receive vitamin D (irradiated ergosterol), when the Ca:P ratio was greater than 2.00. The addition of irradiated ergosterol to the ration of the corresponding groups increased the inorganic phosphorus content to near normal, as reported by Kramer and Howland (11). Added phosphorus, in the form of secondary sodium phosphate, or a change in Ca:P ratio from 1.00 to 0.25, increased the inorganic phosphorus values of the blood serum in

TABLE IV

*Effect of Varying Calcium and Phosphorus Intake, with and without Irradiated Ergosterol, on Inorganic Phosphorus Content of Blood Serum*

Results are expressed in mg. per 100 cc.

Ca:P ratio	Ca-P concentrations					
	Controls			Irradiated ergosterol		
	Low	Medium	High	Low	Medium	High
5.00	2.9	2.4	3.3	5.3	8.1	8.2
4.00	2.5	2.4	3.6	6.8	8.1	7.0
3.00	3.5	4.1	3.8	7.3	7.7	6.9
2.00	6.2	6.3	6.0	8.1	10.5	10.1
1.00	9.2	10.4	8.8	9.3	10.2	9.2
0.50	12.2	10.1	9.6	9.0	10.9	8.1
0.33	11.4	12.2	11.0	8.7	13.0	9.5
0.25	13.5	13.4	11.8	11.5	15.8	11.1

Considerable difficulty was experienced with hemolysis in the samples from some of the high phosphorus groups.

the control groups. The addition of vitamin D (irradiated ergosterol) to the same groups did not appear to affect the phosphorus content of the serum. It is also significant to note that as the proportion of phosphorus to calcium in the ration was increased a more or less progressive increase in inorganic phosphorus content of the serum was noted, irrespective of the presence or absence of the antirachitic factor. These observations are in general in agreement with those recently reported by Kramer and Howland (11). It should be noted that the inorganic blood phosphorus of the majority of the irradiated ergosterol-fed groups in the medium

concentration series was somewhat higher than in the low or high series. We are of the opinion that the increased phosphorus was due primarily to the hemolyzed condition of the serum rather than the effect of phosphorus concentration in the ration.

The calcium content of the blood serum (Table V) was not as much affected as the inorganic phosphorus. All groups, irrespective of the Ca:P ratio or the concentration of these elements in the ration, with the exception of the control groups which received a greater proportion of phosphorus than calcium in their rations, showed a calcium content within the normal range (11). It is of

TABLE V

*Effect of Varying Calcium and Phosphorus Intake, with and without Irradiated Ergosterol, on Calcium Content of Blood Serum*

Results are expressed in mg. per 100 cc.

Ca:P ratio	Ca-P concentrations					
	Controls			Irradiated ergosterol		
	Low	Medium	High	Low	Medium	High
5.00	10.7	13.3	11.1	13.0	11.2	13.4
4.00	9.9	12.4	11.8	12.2	11.6	12.8
3.00	9.3	11.9	12.4	12.5	12.5	11.4
2.00	10.5	10.7	9.2	12.3	11.5	9.5
1.00	9.9	9.6	9.7	11.7	11.9	10.6
0.50	7.8	7.7	7.3	12.7	9.0	10.4
0.33	6.4	9.9	6.6	12.8	12.4	9.4
0.25	5.3	6.1	7.4	11.4	9.9	10.7

especial interest to note, however, that the calcium percentage of the blood serum was reduced as the Ca:P ratio of the ration was changed from 1.00 to 0.25, in the absence of irradiated ergosterol (vitamin D). When vitamin D was added no such reduction in calcium content was noted. These observations are in accord with those previously reported by Bethke, Steenbock, and Nelson (5), and recently by Kramer and Howland (11). The groups which showed a high phosphorus-low calcium content of the blood serum were nervous and excitable. Whether these animals were bordering on tetany we are unable to state.

The results of the ash determinations on the femurs are recorded

in Table VI. These data show conclusively that calcification, especially in the absence of vitamin D, is greatly affected by the Ca:P ratio of the ration. Keeping the per cent phosphorus in the ration practically constant and increasing the per cent calcium, through the addition of calcium carbonate, caused a progressive decrease in the ash content of the femurs. The addition of irradiated ergosterol (vitamin D) to the same rations prevented the occurrence of rickets and improved calcification in all instances. However, the same general decline in per cent bone ash, although less pronounced, was observed by increasing the Ca:P ratio from

TABLE VI

*Effect of Varying Calcium and Phosphorus Intake, with and without Irradiated Ergosterol, on Ash Content of Femurs*

Results are expressed in per cent on a fat-free basis.

Ca:P ratio	Ca-P concentrations					
	Controls			Irradiated ergosterol		
	Low	Medium	High	Low	Medium	High
5.00	22.6	26.9	30.1	50.8	53.8	51.6
4.00	23.6	27.2	32.5	51.3	54.7	52.0
3.00	32.2	36.5	40.2	54.4	57.7	54.2
2.10	42.2	45.1	48.8	56.3	59.9	58.1
1.10	46.4	52.3	51.3	57.4	59.2	59.8
0.50	46.3	50.9	51.6	58.3	58.8	58.3
0.33	47.2	48.5	51.2	56.9	57.3	55.8
0.25	46.6	48.9	48.6	57.3	55.2	55.2

1.00 to 5.00. This observation gives further support to the earlier findings of Bethke, Steenbock, and Nelson (5) that the wider the Ca:P ratio the greater are the requirements for vitamin D. When the Ca:P ratio was changed from 1.00 to 0.25 through the addition of phosphorus, in the form of secondary sodium phosphate, a slight reduction in per cent ash was observed in the medium and high concentration series. The per cent decrease in bone ash was as great in the groups fed irradiated ergosterol (vitamin D) as in the controls. We had anticipated that a ration much higher in phosphorus than in calcium would greatly affect the per cent of bone ash, especially in the absence of the anti-

rachitic vitamin, since in earlier investigations rations of this type were used for the production of experimental rickets. Apparently, in these experiments the per cent calcium in the rations was either too high or the Ca:P ratio was not sufficiently wide to produce changes ordinarily observed on a high phosphorus-low calcium dietary.

Increased levels or concentrations of calcium and phosphorus in the rations exerted a favorable effect on bone ash in the absence of vitamin D, especially when the amount of calcium was equal to or greater than that of phosphorus. The inclusion of the anti-

TABLE VII

*Effect of Varying Calcium and Phosphorus Intake, with and without Irradiated Ergosterol, on Growth of Rats*

Results are expressed in gm.

Ca:P ratio	Ca-P concentrations					
	Controls			Irradiated ergosterol		
	Low	Medium	High	Low	Medium	High
5.00	29	40	42	30	49	44
4.00	30	43	46	53	62	52
3.00	49	72	57	67	104	83
2.00	58	72	81	90	101	85
1.00	54	65	89	86	98	96
0.50	35	37	59	85	84	90
0.33	25	31	28	76	50	44
0.25	15	15	13	74	32	33

rachitic factor (irradiated ergosterol) increased the per cent of ash in all instances. The greatest favorable effects were obtained when the calcium content of the ration exceeded that of phosphorus.

The degree of calcification in the ulnæ and radii in all cases closely checked with the ash determinations.

The average growth of the different groups is presented in Table VII. The data show that growth is greatly affected by the Ca:P ratio of the ration. With few exceptions, better growth occurred when the amount of calcium in the ration exceeded that of phosphorus than when the reverse was true. The rate of gain,



however, progressively declined as the width of the Ca:P ratio increased, irrespective of the presence of vitamin D, or whichever element was in excess. The inclusion of the antirachitic factor (irradiated ergosterol) increased growth regardless of concentration or ratio in all cases. It is evident from the data that the greatest growth occurred at Ca:P ratios of 2.00 and 1.00, regardless of the concentration or per cent of these elements in the ration.

#### DISCUSSION

The data, in general, show conclusively that in the absence of vitamin D the proportion of calcium to phosphorus in the ration, within certain limits, exerts a marked effect upon growth, bone formation, and the percentages of these elements in the blood. Although the concentration or the levels at which calcium and phosphorus were present in the ration had some beneficial effect on growth and bone formation, this effect was not as great as that exerted by the actual proportion between the elements. The inclusion of vitamin D not only tended to stabilize the calcium and inorganic phosphorus concentrations in the blood serum, but made a greater percentage of these elements available for such biological phenomena as calcification and growth. Evidence is also presented showing that the wider the Ca:P ratio the greater are the requirements for vitamin D. With the same amount of irradiated ergosterol, a ration possessing a Ca:P ratio of 5.00 produced bones from 6 to 7 per cent lower in ash than did a similar ration with a Ca:P ratio falling between 2.00 and 0.50.

Since all work bearing on the calcium-phosphorus relationship in calcification brings out the importance of the ratio of these two elements in the ration, it is imperative that these facts be taken into consideration when attempts to evaluate a particular ration or foodstuff for its calcifying properties are made. It would also appear to be essential and desirable, from an experimental standpoint at least, to define our more or less standard rickets-producing rations, or those rations employed for calcification studies, in terms of calcium and phosphorus, as well as kind and proportion of ingredients. Such a procedure would certainly tend towards greater uniformity of results between different laboratories or investigators who draw upon different stocks or sources of supply for the ingredients used in the compounding of their experimental

rations. We know from experience that grains, such as corn, as well as other foodstuffs, vary greatly in their inorganic composition, depending upon such factors as soil fertility, rainfall, climate, variety, etc. The kind of corn-meal, *i.e.* whether it is a meal prepared from the entire corn grain or from the common market "corn-meal" which, in many instances, is devoid of the corn germ, may be a major factor. Data showing great variation in the inorganic composition of the corn grain and commercial corn meals were recently presented by Holmes and Tripp (12).

It also appears that some distinction should be made between calcification studies designed to study the "over-all" effect of a particular foodstuff on one hand and its inherent vitamin D potency on the other. Experiments planned to determine the vitamin D content of foods, like milk, which supply considerable quantities of calcium and phosphorus, by feeding varying amounts of the same, without adjusting the calcium and phosphorus content of the basal ration accordingly, and determining their effect on calcification, in reality do not strictly measure the vitamin D potency of the food under examination. Rather they measure the "over-all" or combined effect of the inorganic elements and vitamin D. Any addition to a ration which makes for a Ca:P ratio approaching 2.00 to 1.00 would of itself make conditions more favorable for calcification and materially reduce the requirements for vitamin D. The actual vitamin D content of a food can only be determined when other inherent factors, such as calcium and phosphorus, are corrected for. Recently Bloom (13) reported that secondary calcium phosphate prevents and cures rickets in rats without vitamin D. This investigator substituted an equivalent quantity of calcium in the form of secondary calcium phosphate for the calcium carbonate in his basal rachitic ration and noted a decided increase in the per cent bone ash. Such a substitution naturally changes the Ca:P ratio from one that induces rickets to one that falls within the optimum value for good bone formation. In our experiments a similar favorable Ca:P ratio gave approximately the same per cent of bone ash (50 per cent). This, however, was not a maximum value since the addition of irradiated ergosterol (vitamin D) made for a 57 to 59 per cent ash content, which, in light of our interpretation, was normal.

It is probable that the form in which calcium and phosphorus

are included in the ration may exert an influence. Experimental work bearing on these points has been reported, but the evidence, in general, is conflicting, and, in the light of present knowledge of the effect of the Ca:P ratio, a great deal of the work needs to be repeated. To what extent the reaction of the ration exerted an effect on calcification we are unable to state. Evidence in this respect is at variance and requires a great deal more work before we can come to an intelligent understanding of the problem.

#### SUMMARY

1. The ratio of calcium to phosphorus in a ration exerts a marked effect on growth, ash content of the bones, and the calcium and inorganic phosphorus values of the blood serum of rats.

2. Increasing the Ca:P ratio from 1.00 to 5.00 causes a progressive decrease in growth, bone ash, and the percentage of inorganic phosphorus in the blood serum. The addition of irradiated ergosterol (vitamin D) to the ration of corresponding groups made for increased growth, ash values, and normal inorganic phosphorus of the blood.

3. When the Ca:P ratio was changed from 1.00 to 0.25, growth was depressed and the ash content of the femurs was slightly decreased at the two highest Ca:P concentrations. The same change in ratios caused a decrease in the per cent calcium and an increase in the per cent phosphorus in the blood serum. Added vitamin D (irradiated ergosterol) increased growth and the ash content, but did not influence the decline in ash values or the increase in inorganic phosphorus of the blood serum. Vitamin D also made for normal calcium values of the blood serum.

4. Increased levels or concentrations of calcium and phosphorus in the ration exerted a beneficial effect on growth and bone ash.

5. The ratio of Ca:P had a greater effect on growth and calcification than the concentration or levels of these elements in the ration.

6. The most favorable Ca:P ratio for growth and bone formation was between 2.00 and 1.00. The vitamin D requirements were at a minimum at these proportions.

7. Further evidence is presented which shows that the wider the ratio of Ca:P the greater are the requirements for vitamin D.

8. The desirability of adjusting the Ca:P relationship in calcifi-

cation studies, and the importance of defining rachitic rations in terms of calcium and phosphorus as well as ingredients, are pointed out.

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# THE DETERMINATION OF IODINE IN BLOOD, FOODS, AND URINE\*

BY EMIL J. BAUMANN AND NANNETTE METZGER

WITH THE COOPERATION OF LEON K. BALDAUF

(From the Laboratory Division, Montefiore Hospital, New York)

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Iodine estimations in which organic matter is destroyed by open combustion have proved very discouraging. Orr (1) investigating the iodine methods suitable for the analyses of foods, soil, blood, etc., and their application to the study of goiter concludes, "It has been made clear that the present methods in use for the estimation of iodine in very minute quantities and in the presence of organic materials are so unsatisfactory as to impede further progress in research. There is need for a standard method of estimation suitable for use by all workers alike that will give results closely approximating to accuracy, or results in which the standard error is constant and known."

Four iodine determinations on a vegetable preparation after combustion in a muffle furnace at 400–450° gave the following results: 0.17, 0.16, 0.23, and 0.25 mg. per kilo. Through the courtesy of the South Carolina Food Research Commission four determinations on a similar preparation were made for us with equally great variation. We have never been able to run satisfactory duplicates even at a temperature as low as 350°. When iodides are added to foods, losses of 10 to 25 per cent or more occur during incineration; the results are irregular and uncontrollable. We find that even in the presence of weak alkali, iodides are lost when heated at comparatively low temperatures.

We therefore turned to the use of a closed system with an absorption train. All the methods of this class that have been described are elaborate, time-consuming, and not very satisfactory. In theory at least the McClendon method (2) in which a Cottrell

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precipitator is used to condense the smoke cloud arising from combustion should be capable of yielding good analyses but the work of assembling the apparatus, of carrying out the combustion, and then of washing out the apparatus with its long absorption train, is so great that not more than one or two combustions can be made in a day. Since McClendon's original paper was published, he has abandoned this procedure in cases in which the amount of iodine present is relatively large and has advocated the use of a muffle furnace for incineration instead of the large combustion tube and Cottrell precipitator (3).

In view of all this we hoped that better results might be obtained by making the combustion in a bomb. For this purpose an Emerson bomb proved fairly satisfactory. About six combustions could be made in a day but the maximum charge could be only a little over a gm. This serious limitation was overcome by burning the substance in a flask in a current of oxygen. We have used this type of apparatus for more than a year. Such a combustion proceeds quietly and comparatively quickly but it gives rise to a smoke cloud which cannot be absorbed by passing through half a dozen or more efficient gas wash bottles. A simple device described by Weber (4), consisting of two funnels placed mouth to mouth with some filter papers between and made gas-tight with paraffin, proved remarkably efficient. With this simple apparatus we have been able to obtain consistent results, using the methods of Leitch and Henderson (5) and of Kelly and Husband (6) for the actual estimation of iodine, somewhat modified to meet our conditions. The accuracy possible is limited by the exactness with which one can measure 0.05 cc. and with the pipette used here, this is about 10 per cent. When 0.002 to 0.01 mg. of iodine is present, the accuracy of the method is 3 to 5 per cent.

The value of this apparatus for incineration of biological material in the estimation of minute amounts of inorganic constituents, and especially of the more volatile ones, will be apparent.

### *Reagents*

1. *Distilled Water*—Add enough potassium permanganate to distilled water to give a purple color and let it stand 24 hours. To 8 liters of such water add about 30 cc. of 50 per cent sodium hydroxide, a little charcoal to prevent bumping, and distil, using

either glass or rubber stoppers for connections and a glass or tin condenser.

2. *Potassium Hydroxide*—A 10 per cent solution of reagent grade kept in a paraffin coated bottle.

3. *Hydrogen Peroxide*—30 per cent Merck's superoxol is distilled *in vacuo* at a temperature below 60°. The peroxide is not allowed to come in contact with organic matter but rubber stoppers are used on the distillation flasks. In an ice box this reagent will keep for months with little decomposition.

4. *Bromine*—Bromine is purified by shaking 50 cc. in a separatory funnel five times with 10 cc. of 0.1 N NaOH. It is then distilled under a little very dilute alkali in an all-glass distilling apparatus or from a retort that fits loosely into a condenser without any stopper.

5. *Sulfuric Acid*—3 and 20 per cent solutions.

6. *Sodium Bisulfite*—10 per cent solution kept in an ice box. It keeps for at least 2 weeks.

7. *Potassium Iodide*—A 30 per cent solution made up the day it is to be used. We have found several of the analyzed grades to yield molecular iodine in solution much more readily than the U.S.P. grade.

8. *Soluble Starch*—A 1 per cent solution.

9. *Methyl Orange*—A 0.01 per cent solution.

10. Pieces of anthracite coal about 1 to 2 mm. in diameter, which are used to prevent bumping and for the rapid removal of bromine from solution (7). These pieces are cleaned by boiling in 10 per cent nitric acid, washing free of acid, and then heating to glowing in a platinum or silica dish.

11. Sodium thiosulfate solution, 0.001 N, standardized against potassium iodate solution as follows: 2 cc. of 0.001 N potassium iodate solution are measured with a calibrated pipette into a 50 cc. conical flask. Add 8 cc. of water, 0.6 cc. of 3 per cent sulfuric acid, and 0.5 cc. of 30 per cent potassium iodide. Titrate until the yellow color becomes faint, add 0.25 cc. of starch solution, and continue titration until the blue color disappears. A 2 cc. micro burette graduated in 0.01 cc. and delivering a drop not larger than 0.02 cc. is used. In an ice box this solution will not change significantly for at least 2 weeks.



*Apparatus<sup>1</sup>*

Fig. 1 shows the system used. It consists of a 3 or 5 liter balloon flask, *W*, fitted with a rubber stopper through which enter (1) a Pyrex glass tube, *e*, 5 to 6 mm. inside diameter, flared at the bottom, through which oxygen passes into the flask, (2) an outlet tube, *G*, and (3) two chromium steel rods, *d* and *d'*, about 3.5 to 4.0 mm. in diameter. One of these rods is bent at the bottom into a ring and serves as a holder for the vessel in which the charge is burned as well as for one of the terminals for conducting a small

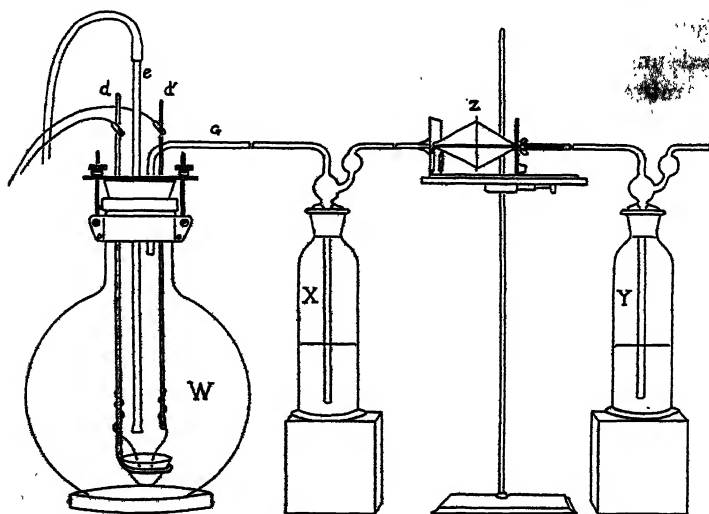


FIG. 1. Set-up of the apparatus used for iodine determinations. *W* is the balloon flask fitted with a rubber stopper through which enter Pyrex glass tube, *e*, two chromium steel rods, *d* and *d'*, and an outlet tube, *G*. *X* and *Y* are ordinary Drexel wash bottles. *Z* consists of two funnels placed mouth to mouth so as to be gas-tight, which serve as a smoke condenser.

electric current. The charge is placed in a porcelain crucible or iron dish of suitable size and a platinum wire, 0.025 mm. in diameter and 3 to 4 cm. long, connects the terminals and is brought in contact with the charge. For 10 cc. of dried blood a wide mouthed crucible with a top diameter of 35 mm. is used. The clamp furnishes a convenient means of fastening the rubber stopper

<sup>1</sup> Constructed by Mr. H. Hawker, mechanic to the laboratory.

into the combustion flask but is not essential; wire may be used instead. The clamp consists of a metal collar around the flask and a metal plate on the rubber stopper, through which the tubes and rods pass with generous openings, so that the terminals,  $d$  and  $d'$ , do not come into contact with the plate. It is fastened to the collar by means of two thumbscrews. The ordinary Drexel wash bottles,  $X$  and  $Y$ , are 250 cc. in size and the smoke condenser,  $Z$ , consists of two 6.5 cm. funnels placed mouth to mouth with four pieces of closely woven analytical filter paper between them (Schleicher and Schüll No. 589, blue ribbon, or Whatman No. 42, etc.).<sup>2</sup> The funnels are held firmly in a suitably constructed clamp or frame and made gas-tight by coating the edges with clean paraffin of high melting point. The parts of the set-up are connected as closely as possible with gum tubing.

An exceptional degree of cleanliness is necessary. Apparatus should be washed with cleaning solution, rinsed with distilled water, and finally with the doubly distilled water.

To insure good contact, the iron electrodes with the platinum wire are dipped into boiling 10 per cent hydrochloric acid for a moment and rinsed with water as above.

#### *Preparation of Sample for Analysis*

**Blood**—10 cc. or more are pipetted into small flat evaporating dishes and dried in an oven at 80–85° for 6 hours. The dried mass is broken up by hand and the part that adheres to the dish is scraped off with a sharp edged steel spatula and all brushed into a porcelain crucible.

Food is hashed and similarly dried at 80–85°.

Urine is dried on a water bath with enough caustic alkali to make it strongly alkaline. Substances containing comparatively little organic matter, like urine, will not burn easily. They are

<sup>2</sup> Washing a few hundred cc. of water through the best grades of filter paper gives a filtrate that contains a considerable amount of organic matter. We have therefore found it necessary to wash the best paper obtainable with a large volume of 0.01 per cent KOH several times, then four times with distilled water, and once or twice with doubly distilled water. The paper is sucked nearly dry on a Buchner funnel and dried in an oven at 80–90°. This paper is used for the smoke absorber. Filtrations are all made through glass Buchner funnels with fused-in discs of sintered glass of the finest porosity.

therefore mixed with a little iodine-free dry powdered sugar, salicylic acid, or other material rich in carbon.

### *Analysis*

*A. Oxidation*—In the wash bottles *X* and *Y* place 150 cc. and 100 cc. of water respectively. When working with 0.001 to 0.004 mg. of iodine, 0.05 cc. of 10 per cent potassium hydroxide is added to *Y*; with 0.005 to 0.03 mg. of iodine, 0.4 cc. of 10 per cent potash is used. The crucible containing the charge is set into the chromium steel ring, the platinum ignition wire being adjusted so that good contact is made with the charge. The rubber stopper (with its contained parts) is set carefully into the flask and fastened; and the flask, wash bottles, and smoke condenser connected as shown in Fig. 1. Pass oxygen through the apparatus for a minute or so at a rate of about 600 cc. per minute, avoiding sudden changes in the rate that might break the paraffin seal of the smoke condenser. Then ignite the charge; the material bursts into flame. Continue passing oxygen through until the charge is burned up and the white fumes remaining in the flask have been sent through the smoke filter. Disconnect the chain beginning at the distal end.

*B. Preparation for Analysis*—Cut the smoke condenser apart with scissors and drop the filter papers into an 800 cc. beaker; rinse the funnels into it. Place the crucible in the same beaker, rub the sides well with a policeman, wash the stopper and its tubes and rods into the beaker, and finally pour the contents of the wash bottles into the same beaker and wash these also. The washings, which should be about 450 cc. or less, are heated to about 40° and filtered through a 45 mm. glass Buchner funnel with disc of sintered glass of finest porosity and washed into a 1 liter evaporating dish. At this point add 0.35 cc. of 10 per cent potassium hydroxide when working with 0.001 to 0.004 mg. of iodine (with 0.005 to 0.03 mg. of iodine use 0.4 cc. of potassium hydroxide) and then 5 cc. of 30 per cent hydrogen peroxide or more depending on the yellowness of the filtrate.<sup>3</sup> Evaporate on a water bath to 3 to 4 cc., filter off the iron hydroxide, etc., through a 30 mm. glass Buch-

<sup>3</sup> This is a convenient point to interrupt the analysis, if necessary, as the peroxide can carry on its action in the interval. A glass-stoppered conical flask is a good vessel for storing the solution.

ner funnel, and collect filtrate and washings in a 100 cc. beaker. Concentrate on a water bath or hot plate to 1 to 2 cc., transfer to a 25 cc. platinum dish, and concentrate to a few cc. Add 0.05 cc. of 10 per cent potassium hydroxide (with 0.005 to 0.03 mg. of iodine use 0.7 cc.) and continue evaporating *just* to dryness. Place in a vacuum desiccator overnight.

In the analysis of urine or of foods where 10 gm. or more of material are used, it is necessary to extract at this point with about 8 cc. of 80 per cent ethanol. (This is purified by distilling from alkali.) The salts are filtered off and washed five or six times with 2 cc. portions of 80 per cent alcohol. The filtrate and washings are evaporated *just* to dryness in a 25 cc. platinum dish.

A small amount of organic matter is still present, which must be removed completely before the determination may be made. This has usually been accomplished by incinerating, Stolte fashion, for a minute or less or until the material just fuses. Large losses not infrequently occur at this point. We have, therefore, preferred to carry out the final combustion in an inclosed system (Fig. 2) and for this purpose have used a wide mouthed, 1 liter conical flask fitted with a rubber stopper through which pass (1) an entry tube reaching to about 4 cm. from the bottom and an exit tube, both of which are 5 to 6 mm. in diameter, (2) two chromium steel rods 2.5 mm. in diameter, which act as terminals; one of these is bent into a circle at the bottom and serves, with a short cross strip of iron, as a support for a heating unit and the platinum dish. The heating unit is made from 4 feet of 0.025 mm. platinum wire wound into a coil about 1.5 mm. in diameter, which is arranged in a spiral and fastened at many points on a 4 cm. perforated porcelain filtering plate. The heating unit is placed on the iron ring and the ends of the coil fastened to the terminals; another perforated porcelain plate (from the bottom of a Gooch crucible) covers the coil and on this the platinum dish is set, avoiding direct contact with the terminals or heating coil. The rubber stopper with its fittings is then set in the conical liter flask, connected to a very small gas wash bottle containing 15 to 20 cc. of water. Oxygen is passed through for a few seconds. Connect the terminals with a rheostat, and slowly increase the current going through the coil so that sputtering is avoided, until the coil glows quite brightly. Continue heating for 10 to 15 minutes. Turn off the current,

disconnect the apparatus, and cool the flask in running water. Wash into a 250 cc. beaker and evaporate on a hot-plate or water bath to 1 to 2 cc., shaking if necessary to prevent drying in spots. When 0.001 to 0.004 mg. of iodine is present, filter through a small glass filter, transfer to a 30 cc. conical flask, and reduce the volume to 7 to 8 cc., while with 0.005 to 0.03 mg. of iodine transfer to a 50 cc. flask, concentrating the volume to 15 cc.

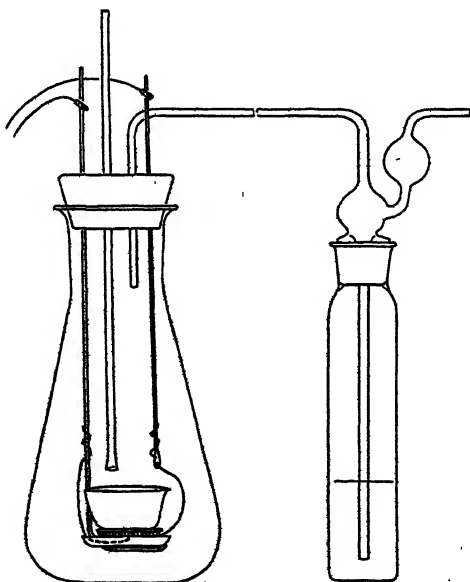


FIG. 2. The inclosed system used for the final combustion consists of a 1 liter flask fitted with a rubber stopper through which pass an entry tube and two chromium steel rods which act as terminals, one of which serves as a support for the heating unit and platinum dish.

**C. Determination of 0.001 to 0.004 Mg. of Iodine<sup>4</sup>**—Add a drop of methyl orange solution and just acidify with 3 per cent sulfuric acid (about 0.3 cc. is needed); add 0.7 cc. of 3 per cent sulfuric acid, 0.4 cc. of 10 per cent sodium bisulfite, and a piece of washed coal.

<sup>4</sup> The solution may be very slightly yellow due to the presence of a little iron; this does no harm. If, however, the color is due to organic matter, indefinite end-points are obtained and the results will be high.

Boil for exactly 3 minutes and cool at once. Add 2 or 3 small drops of bromine and shake until the fluid is orange-yellow. Boil off the bromine and continue concentration until the volume is reduced to 2.5 cc.  $\pm$  0.5 cc., cool, and remove the coal with a pair of slender forceps. Add 0.2 cc. of (freshly prepared) 30 per cent potassium iodide and 2 drops of a 1 per cent soluble starch solution, and titrate immediately with 0.001 N thiosulfate with a 0.2 cc. pipette graduated in 0.01 cc. according to Leitch and Henderson.

*D. Determination of 0.005 to 0.03 Mg. of Iodine*—Add a drop of methyl orange and acidify with 20 per cent sulfuric acid; add 0.8 cc. of 20 per cent sulfuric acid, 0.4 cc. of sodium acid sulfite, and a piece of coal. Boil for exactly 2 minutes, cool at once, add 2 or 3 small drops of bromine, and shake until the solution is a deep orange-yellow. Boil until the bromine color has disappeared and then for exactly 2 minutes more. Add a small pinch of salicylic acid (10 mg.), cool, and remove the coal with forceps at once. Add 0.5 cc. of 30 per cent freshly prepared potassium iodide and 0.25 cc. of starch solution and titrate at once with 0.001 N thiosulfate with a 2 cc. micro burette graduated in 0.01 cc. and delivering a drop not larger than 0.02 cc.

*Notes on Method*—Each step of the Kelly and Husband and the Leitch and Henderson methods that we have used has been thoroughly tested and adapted to our conditions. The results of this examination as well as other findings will be discussed in these notes.

1. Alkali iodides are more volatile than has usually been supposed. Significant losses occur when they are heated in an oven at 85°. For example, portions of potassium iodide equivalent to 0.0102 mg. of iodine were heated in conical flasks in an oven at 85° for 24 hours. The iodine was then estimated with the following recoveries: 0.0093, 0.0087, 0.0077, 0.0089 mg. When heated under similar conditions or on the water bath for half an hour, with 0.1 gm. of potassium hydroxide, no iodine was lost. Even when heated in a crucible for 2 minutes with 0.1 gm. of potassium hydroxide, according to Stolte, no loss of iodine occurred starting with 0.001 mg. or 0.01 mg. of iodine. Since the hydroxide added could easily be converted to carbonate in the course of the analysis, we made similar tests, with sodium car-

bonate in place of potassium hydroxide. Heated just to dryness on the water bath with 0.1 gm. of sodium carbonate, only 0.0090 mg. of iodine was recovered from 0.0102 mg. When the heating on the bath was continued for half an hour, only 0.0076 mg. of iodine was recovered.

From figures such as the above, which have been obtained many times, we may conclude that heating iodides alone or with carbonate at temperatures below  $100^{\circ}$  for a comparatively short time results in serious losses of iodine while in the presence of caustic alkali such treatment does not result in loss of iodine.

2. Because the halides are converted largely to chlorates, bromates, and iodates during the combustion, similar tests were made with potassium iodate. When heated in the oven at  $85^{\circ}$  or on the water bath, even without the addition of alkali, no iodine was lost.

3. Since chlorates and bromates must be reduced to the corresponding halide before the determination can be made, rather large amounts of sodium bisulfite are used. For this reason and because the composition of the bisulfite is apt to vary, a separate blank test is required each time new sulfuric acid or sulfite solution is made up so that the proper proportions of both can be used. Put 0.4 cc. of 10 per cent sodium bisulfite, 0.7 cc. of 3 per cent sulfuric acid, a piece of coal, and 5 cc. of water into a 30 cc. flask, boil for 3 minutes, cool, and add 2 drops of bromine. Shake until deep yellow; boil until the volume is reduced to 2.5 cc. Cool and add 0.2 cc. of 30 per cent potassium iodide and 2 drops of starch.

If a permanent yellow is not obtained rapidly on adding bromine and shaking, too much bisulfite is present. If after oxidation with bromine a blue color develops in less than 2 or 3 minutes after adding potassium iodide and starch, there is too much acid present.

4. With 0.001 to 0.004 mg. of iodine, the volume in which the final titration should be made must be between 2 and 3 cc. In this volume, the amount of 30 per cent potassium iodide may vary between 0.2 and 0.4 cc., while that of 3 per cent sulfuric acid may vary between 0.6 and 0.9 cc. We have used 0.2 cc. and 0.7 cc. respectively.

5. With 0.005 to 0.03 mg. of iodine, the titration should be made in a volume of 10 to 15 cc. In this volume the amount of 30 per cent potassium iodide and 20 per cent sulfuric acid may both vary

between 0.5 and 0.1 cc. We have used 0.5 cc. and 0.8 cc. respectively.

To test the reliability of the method, we have estimated the amount of iodine in pure iodides and iodates, with results that approach the theoretical as closely as our apparatus permits. In addition, amounts of potassium iodide varying from 0.001 to 0.01 mg. of iodine have been dried down with 10 cc. of mixed human blood and good recoveries of the added iodine have been obtained. Results of twelve such analyses are presented in Table I.

It must be emphasized that the estimation of iodine in blood, foods, etc., by any method requires a most exacting technique and

TABLE I  
*Recovery of Iodine from Mixed Human Blood*

Present in 10 cc. of blood	I <sub>2</sub> added (as KI)	Total I <sub>2</sub> found	Added I <sub>2</sub> recovered
$\gamma$	$\gamma$	$\gamma$	per cent
0.86	1.02	1.85	97
1.00	1.02	2.00	98
1.00	1.02	2.00	98
1.08	2.04	3.05	97
1.00	10.18	11.25	101
1.00	10.18	11.01	98
1.00	10.18	11.00	98
1.00	10.18	10.75	96
1.00	10.18	10.45	93
1.00	10.18	10.55	94
0.88	10.18	10.78	97
0.88	10.18	11.00	99

that the danger of contamination is very great, scrupulous care being necessary to prevent it. It is necessarily an elaborate and delicate operation but we have found that in skilled hands a more rapid and controlled combustion can be obtained with this comparatively simple and easily assembled apparatus than by open combustion methods and a better economy of time and more accurate results are possible than by the closed combustion methods now in use.

*Addendum*—Since this paper was submitted for publication, Carns (8) has described a somewhat similar but much more elaborate incinerating apparatus in which the products of combustion are collected in an absorption train that includes a Cottrell precipitator.



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## FACTORS IN FOOD INFLUENCING HEMOGLOBIN REGENERATION

### II. LIVER IN COMPARISON WITH WHOLE WHEAT AND PREPARED BRAN\*

By MARY SWARTZ ROSE AND LAN-CHÊN KUNG

*(From the Nutrition Laboratory, Teachers College, Columbia University,  
New York)*

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In studying the relative value of certain cereals as sources of material for hemoglobin regeneration in rats made anemic by exclusive milk feeding, Rose and Vahlteich (1) adopted the method of comparing the hemoglobin increase in a 6 weeks period to determine the relative efficiency of the foods used. In connection with this study Vahlteich (2) found that the amount of hemoglobin regenerated on feeding 1 gm. of beef liver daily was definitely less than that from feeding 1.5 gm. of whole wheat, containing approximately the same amount of iron (0.05 mg.). As liver contains more copper than whole wheat, copper could not be regarded as the limiting factor. This was a surprising result in view of the report by Waddell, Elvehjem, Steenbock, and Hart (3) of excellent results with the ash of liver in amounts to yield 0.5 mg. of iron per day. Although these authors speak also of the unmistakably high value of beef liver in its natural state, a curve representing five or six animals fed dried liver at a level to yield 0.15 mg. of iron shows a rise to only 8 or 9 gm. of hemoglobin per 100 cc. of blood in 6 weeks and a level of 12 gm. was attained only in about 4 months. Shortly after Vahlteich's work was completed Sheets and Frazier (4) published a curve for six animals fed 0.25 mg. of iron 6 days per week in the form of calf liver which showed a rise to less than 10 gm. of hemoglobin per 100 cc. of blood in 6 weeks, and failure to reach normal level in 16 weeks. Vahlteich<sup>1</sup>

\* Based on a thesis presented by Lan-Chên Kung in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> Private communication.

has also fed dried liver to yield 0.1 mg. of iron daily to eight rats with average rise to about 7 gm. of hemoglobin per 100 cc. of blood in 6 weeks. Further investigation seemed most desirable, especially in view of the relatively high iron content of liver and its richness in other minerals and vitamins, all of which make it an important food quite aside from its long recognized value as a source of proteins of excellent quality.

McCay (5) investigating the influence of some organic factors such as proteins and fats on anemia of hemorrhage, found that rats made anemic by cardiac bleeding and placed on a diet of lard, sucrose, and Osborne and Mendel's salt mixture (6) with albumin, casein, and liver as three different sources of protein, regenerated more hemoglobin on liver than on the albumin or casein, even though each was supplemented with ferric citrate to make the iron content of the three diets identical. To induce anemia, an average of 2.25 gm. of hemoglobin had to be removed in case of liver-fed rats as compared with 1.42 gm. from rats on the casein diet and 1.79 gm. on the albumin diet. The difference did not appear to be due to difference in the protein content of the diets. No special attention was at that time paid to copper, which might be a factor, liver being rich in this element while casein and albumin would probably contain little or none.

Studies of the possible influence of vitamins on hemoglobin regeneration have as yet yielded no conclusive results. Guha and Mapson (7) did not regard vitamin B as a factor, but found vitamin G deficiency to be followed by a diminution in the number of red blood cells, and the anemia was readily cured by autoclaved yeast. Copper, glutamic acid, extra acid, and alkaline hematin were without effect.

Jencks (8) in studies of hemorrhagic anemia induced by withdrawing one-third of the blood at one time, found that 0.5 gm. of dried brewers' yeast, 10 cc. of orange juice, or 5 cc. of tomato juice induced regeneration better than casein, butter fat, or starch, but possibly mineral factors as well as vitamins contributed to her results.

One of the difficulties in comparing the efficacy of different substances for hemoglobin regeneration lies in the possibility of potential hemoglobin-building material already present in the body complicating the situation. Robscheit-Robbins (9) stresses

emphatically the enormous amount of reserve material found in the dogs used by Whipple and herself in their investigations of anemia of hemorrhage. McCay (5) also called attention to this in connection with his bleeding experiments. According to Cook and Spilles (10) the iron content of the spleen of the rat can be reduced from 2.10 mg. per gm. of spleen at birth to 0.42 mg. per gm. at 9 weeks by feeding a diet of milk and rice after the rats are weaned at 21 days of age, but only four rats were used in each case and the method depends for its accuracy upon expulsion of iron from the spleen by its contraction when the animal is killed with ether; and there are no data included to prove that the blood would then be entirely removed.

The amount of iron in the rat body at different stages of the life cycle has been found by Smythe and Miller (11) to be highest at birth, 0.0055 per cent of the body weight. It then drops to 0.0026 per cent at 20 days, rises to 0.0043 per cent at 100 days, and for adults on commercial calf meal diet averages about 0.0051 per cent. Rats apparently do not have an exceptionally high reserve of iron at birth.

Possible storage of copper must be taken into account in studies of iron utilization. Livers of the fetus and new-born of a number of species have been found very rich in this element in comparison with livers of adults. In case of the rat, Cunningham (12) reported 12.5 mg. per kilo of dry matter for livers of adults and 70 mg. for livers of the new-born. There is a steady drop in the percentage of copper in the liver from birth onwards. The high storage in the new-born would seem to be used to tide the young animal over the lactation period, as the low copper content of milk is now well known (13). The ease with which anemia is induced by milk feeding appears to depend upon a low content of this element and the elimination of available body stores.

#### EXPERIMENTAL

The experiments reported in this paper were accordingly designed to find out whether the apparently poorer utilization of the iron from liver was due to storage of the element in some form other than hemoglobin or whether the apparent advantage of wheat as a source of iron was a real one. The first step was to deplete normal growing animals of known heritage to a practically uniform state of anemia by milk feeding. The second step was to

feed the depleted animals amounts of iron in the form of (1) whole wheat, (2) prepared bran, (3) liver, or (4) a solid diet supplemented by a ferric citrate solution which would insure complete hemoglobin regeneration in 6 weeks. The third step was to take away the iron-containing supplements and deplete the animals by bleeding to the same level of anemia to which they had previously been reduced by milk feeding, general undernutrition and further hemoglobin regeneration being prevented by milk feeding. Finally the animals thus made anemic were to be killed and the iron content of their bodies determined.

### *Depletion Period*

Rats 4 weeks old, weaned from mothers fed Sherman's Diet 13 (consisting of two-thirds whole wheat and one-third whole milk powder plus sodium chloride equal to 2 per cent of the weight of the wheat) supplemented by meat and lettuce, were placed on a diet consisting solely of fresh certified milk. After 3 weeks on this diet the hemoglobin content of the blood was determined at approximately weekly intervals. The Newcomer method (14) with the same Newcomer disc was employed throughout. Freely flowing blood from the tail was always used. Duplicate samples of 0.025 cc. each were drawn into pipettes and discharged directly into 10 cc. portions of 1 per cent hydrochloric acid. In 5 to 6 weeks the hemoglobin of most of the animals fell below 7 gm. per 100 cc. of blood. All the rats were between 63 and 78 days old when they were ready for the second phase, which will be referred to as the feeding-up period. Any animal whose hemoglobin did not drop below 7 gm. per 100 cc. of blood by the 11th week of age was discarded, as animals beyond this age are depleted very slowly if at all.

### *Feeding-Up Period*

Five different lots were used, consisting of from twelve to nineteen rats each. For two lots, daily supplements to the milk diet were 6.5 gm. of whole wheat and 1.8 gm. of prepared bran, each furnishing 0.2 mg. of iron; for two other lots, 1.6 gm. of air-dried liver and 3.6 gm. of prepared bran, each furnishing 0.4 mg. of iron.

Since a diet of fluid milk only has been criticized as perhaps not

entirely suited to the digestive tract of the rat, a fifth group of seventeen animals was for comparison put on a modified Osborne and Mendel diet. It consisted of casein, 20 per cent; butter, 7 per cent; cod liver oil, 1 per cent; Osborne and Mendel's salt mixture (6) without ferric citrate, 4 per cent; and corn-starch, 68 per cent. A part of the corn-starch was used to absorb an alcoholic extract of brewers' yeast which served as the source of vitamins B and G. As it was desired to keep the iron content of this preparation as low as possible, only glass and porcelain were employed during the operations. Upon analysis, this diet, which will be referred to as Diet K, yielded 1.5 mg. of iron per 100 gm. of total food. It was given *ad libitum* but records of food con-

TABLE I  
*Iron Content of Foods*

Food	Fe per 100 gm.
	<i>mg.</i>
Whole wheat (air-dry).....	3.1
Prepared Bran I (air-dry).....	12
"    "    II* (air-dry).....	12
Dried liver (air-dry).....	25
Milk*, † (air-dry).....	0.08
Diet 13 " .....	2.5
" K " .....	1.5
Ferric citrate solution† (air-dry).....	26

\* Analyzed by Vahlteich.

† Mg. per 100 cc.

sumption were kept in all cases. Six times per week each animal on this diet received an aqueous solution of ferric citrate (U.S.P.) adjusted to yield the equivalent of 0.22 mg. of iron daily, in combination with a few gm. of food. The rest of the food was withheld until this was consumed.

Eleven negative controls were kept on milk only and most of them died in an anemic state before the end of the experiment. Those that survived were chloroformed when their litter mates on the experimental foods were killed.

In this period food records were kept daily and weight records weekly. Fresh milk carefully measured in a small graduated cylinder was given daily. The unconsumed portion was also

*Average Weight Changes, Food Consumption, and Hemoglobin Production for Each Group during 6 Weeks Feeding-Up Period*

Diet	No. of cases		Weight at start		Gain in weight		Food consumption			Hemoglobin at start, per 100 cc. blood	Total hemoglobin increase				
	♂	♀	♂	♀	♂	♀	Milk	Estimated Fe from milk	Fe from supplement		Per 100 cc. blood (deter-		Per rat (estimated)		Per gm. gain in weight (estimated)
											♂	♀	♂	♀	
Milk + 6.5 gm. whole wheat.....	12	2	110	105	100	44	1230	1.1	8.4	5.8	10.4	1.5	1.1	0.014	0.023
" + 1.8 " prepared bran.....	9	5	98	90	87	62	1830	1.4	8.4	5.4	10.7	1.3	1.1	0.016	0.019
" + 1.6 " dried liver.....	14	5	101	100	130	86	2524	2.1	16.8	5.9	10.3	1.7	1.5	0.013	0.017
" + 3.6 " prepared bran.....	8	10	124	97	83	69	1961	1.6	16.8	5.9	10.7	1.7	1.3	0.016	0.019
Diet K.....	8	9	98	87	142	106	491*	7.4†	9.2†	6.2	11.2	1.9	1.6	0.014	0.017

Gm. of dried food.

Iron from dried food.

Iron added as ferric c

measured and noted, but discarded. Distilled water was accessible to the rats at all times. The food supplement under investigation for its hemoglobin-producing capacity has been calculated in terms of daily dosages but for convenience it was fed three times a week.

Iron analyses were made in triplicate by the Zimmermann-Reinhardt method as modified by Rose, Vahlteich, Robb, and Bloomfield (15). The iron content of the foods used is shown in Table I and weight changes, food consumption, and hemoglobin production in Table II.

### *Bleeding Period*

The third part of the experiment started at the end of the 6 weeks feeding-up period, when the hemoglobin content of the blood of each rat had reached the normal value of about 16 gm. per 100 cc. of blood. The iron-bearing supplement or Diet K was then withdrawn and the rats again placed on an exclusive milk diet. As controls, normal animals, litter mates of some of the experimental animals, fed Sherman's Diet 13 after weaning, were also transferred to milk only at about the same age. To render animals as old as these anemic by a milk diet is hardly possible, hence recourse was had to blood withdrawal by cardiac puncture twice every week. Each time before the animals were bled, hemoglobin readings of the tail blood were made, generally the same day; if this were impossible, the day before. The technique of cardiac puncture was essentially the one used by McCay<sup>2</sup> with a few slight modifications. The left chest region of the rat was wiped with alcohol for sterilization, moistening the hair sufficiently to render it unnecessary to do any clipping. The animal was placed on its back by an assistant. The experimenter then carefully palpated the pulse and after locating the ventricle, quickly thrust in the previously sterilized needle, attached to a syringe. The desired amount of blood was 1 to 2 cc., but it was not always possible to obtain that amount. The blood was delivered directly from the syringe into 10 cc. graduated test-tubes filled with 7 cc. of 0.1 N hydrochloric acid. By use of the hemoglobin reading

<sup>2</sup> Special thanks are due to Dr. C. M. McCay for helpful suggestions on the technique of cardiac puncture.



TABLE III  
Average Weight Changes, Food Consumption, and Hemoglobin Production during Bleeding Period

Diet	No. of cases		Length of period*		Weight at start		Change in weight		Milk consumed per gm. rat per day		Fe from milk (estimated)†		Hemoglobin, per 100 cc. blood				Hemoglobin removed		Hemoglobin removed per gm. rat†
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Milk + 6.5 gm. whole wheat	9	2	28-42 (35)	30-49 (39)	215	151	+28	+9	0.25	0.30	1.5	1.4	16.4	15.8	5.7	5.9	0.94	1.21	0.0044
Milk + 1.8 gm. prepared bran	8	4	21-63 (36)	21-57 (34)	176	145	+20	-27	0.27	0.24	1.3	1.9	16.0	16.4	5.7	5.6	0.85	0.77	0.0048
Milk + 1.6 gm. dried liver	8	2	35-51 (43)	42-44 (43)	231	207	+8	+18	0.25	0.27	2.1	2.0	16.0	16.0	5.5	5.7	1.09	1.30	0.0063
Milk + 3.6 gm. prepared bran	5	7	27-47 (37)	31-54 (40)	211	161	-7	-6	0.24	0.26	1.5	1.4	17.0	16.7	5.5	5.5	1.12	1.33	0.0054
Diet 13	7	4	32-55 (41)	45-61 (50)	248	157	-10	+13	0.25	0.26	2.0	1.7	16.8	16.0	5.8	6.1	1.48	1.24	0.0059
" K	7	4	28-47 (34)	28-45 (38)	230	180	-22	-20	0.20	0.24	1.3	1.3	17.3	17.4	5.6	5.6	1.29	1.20	0.0056

\* Averages are given in parentheses.

† 0.08 mg. per 100 cc. of milk.

‡ Based on initial weight. The upper figure of each pair expresses the result for the males; the lower, that for the females. For iron removed as hemoglobin, calculated as per cent of body weight, see Table IV.

just prior to the bleeding, the amount of hemoglobin withdrawn was calculated from the amount of blood removed.

The rats were chloroformed when their hemoglobin content decreased to 6 gm. per 100 cc. or slightly less. The data reported here for the bleeding period include only those animals that had reached that level at death. The length of time required for this depletion varied from 3 to 8 weeks, the majority of the rats requiring 5 to 6 weeks.

To determine the influence of the milk diet during the bleeding period, a number of rats which had been brought back to the normal on the various supplements were subsequently maintained on milk alone for several months. In all cases the hemoglobin declined so slowly that the fall was hardly detectable from week to week, although appreciable after a few months. For example, a rat given 3.6 gm. of bran daily during the feeding-up period was kept on milk exclusively for 23 weeks, during which time the total fall of hemoglobin was from 15 to 10 gm. per 100 cc. of blood. Such results seem to justify the assumptions that for the duration of the bleeding period the hemoglobin of the animals was neither increased nor diminished by the milk diet and that the amount of hemoglobin withdrawn depended on whatever reserves had been acquired during the feeding-up period. Weight changes, food consumption, and hemoglobin production during the period are shown in Table III.

#### *Determination of Iron Content of Bodies of Depleted Rats*

The bodies of the depleted rats were analyzed for their iron content by the Zimmermann-Reinhardt method, the alimentary tracts being first removed to exclude any food residues which might be present in the digestive tract. Since the ash of the rat body fuses easily, it was necessary to keep the temperature of the muffle as low as possible; then the temperature was not high enough to ignite all of the small amount of carbon found in the bones and repeated extraction was necessary. On account of the large amounts of calcium and phosphorus present, hydrochloric acid was used for extracting. The ash was dissolved in 25 cc. of 12 M hydrochloric acid, filtered through small funnels into 100 cc. volumetric flasks, and washed with approximately 30 cc. of water. The filter paper with the residue was returned to the

TABLE IV  
*Iron Removed As Hemoglobin and Iron Content of Body at End of Bleeding Period*

Diet	No. of cases		Weight minus digestive tract				Total Fe intake*				Fe removed as hemoglobin						Fe of body minus digestive tract			
											Total		Per cent of body weight minus digestive tract		Per cent of intake		To- tal†	Per cent of body weight	Per cent of Fe intake	
	♂	♀	♂	♀	gm.	mg.	♂	♀	mg.	mg.	♂	♀	♂	♀						
	♂	♀	gm.	mg.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.				
Milk + 6.5 gm. whole wheat.....	9	2	197	141	11.0	10.3	3.2	4.1	0.0017	0.0029	29	38	3.80	0.0021	35	65				
" + 1.8 " prepared bran.....	8	4	162	128	11.2	13.9	2.8	2.6	0.0018	0.0022	25	23	3.00	0.0020	27	52				
" + 1.6 " liver.....	8	2	212	199	20.9	20.7	3.6	4.4	0.0018	0.0022	17	21	3.50	0.0022	18	39				
" + 3.6 " prepared bran.....	5	7	187	143	19.7	19.5	3.9	4.5	0.0020	0.0031	20	23	4.00	0.0019	20	38				
Diet 13.....	7	4	216	146			4.9	4.2	0.0023	0.0030			4.20	0.0022						
Milk only†.....	4	7	104	69	1.2	1.7							1.80	0.0022						
Diet K.....	7	4	192	160	18.5	17.0	4.3	4.0	0.0023	0.0025	23	24	3.50	0.0020	20	43				

\* Intake from milk estimated from analyses of milk fed. For analyses of other foods see Table I.

† Differences between sexes not significant.

‡ Hemoglobin fell in periods ranging from 24 to 106 days from an average of 6.0 at start to 3.2 or below at end.

original dish and again ashed. The last traces of carbon were easily removed in this way. This ash was dissolved in 2 to 3 cc. of hydrochloric acid, added to the original filtrate, and the solution made up to volume. Duplicate samples of 40 cc. each were used for titration. The high acidity made it necessary to titrate in a volume of 600 cc. The rest of the procedure followed exactly that used for the foods (15). As a check on the accuracy of the method, iron wire solutions of known strength have been added to quantities of calcium and phosphorus comparable to those found in the rat, and the resulting solution evaporated to dryness and ashed. The iron was quantitatively recovered from this ash. Similarly iron wire solution was added to 10 cc. of rat ash solution and the iron quantitatively recovered. Total iron intake, iron removed as hemoglobin, and iron found in the body at death are shown in Table IV.

#### DISCUSSION

##### *Feeding-Up Period*

Upon addition of the supplements to the milk diet, or upon replacing the milk diet by Diet K, the anemic animals began at once to gain in weight and the hemoglobin content of the blood increased rapidly (see Table II). Complete hemoglobin regeneration took place as expected by the end of the 6 weeks feeding-up period, the average on each diet ranging from 16 to 17 gm. per 100 cc. of blood. The rate of regeneration varied somewhat, being most rapid for the animals on 3.6 gm. of bran (0.4 mg. of iron), as will be seen by reference to Fig. 1. By the end of the 3rd week their hemoglobin had already reached the normal level of 16 gm. per 100 cc. of blood. Preliminary trials indicated that animals fed only 0.8 gm. of dried liver could not be brought to the normal hemoglobin level in 6 weeks. It would seem that 0.2 mg. of iron daily, when copper is adequately supplied, may be regarded as adequate for normal regeneration in 6 weeks if taken in readily available form, and that amounts in excess of this, up to 0.5 mg., may cause a slight acceleration of the rate. To get the same results as with 6.5 gm. of whole wheat or 1.8 gm. of bran, each of which furnished only 0.2 mg. of iron, it was found necessary to increase the dosage of the dried liver to 1.6 gm. daily, yielding 0.4 mg. of iron. The need of more iron in the form of

liver to get the same rise in hemoglobin as with wheat or bran appears to be in accord with Elvehjem's recent report (16) regarding the utilization of inorganic and organic iron in the body. He fed hematin in comparison with ferric chloride to anemic rats and found recovery slower and less complete. The amount found in the liver after 3 weeks of feeding was also less on the organic iron. The more rapid regeneration on 3.6 gm. of bran as compared with

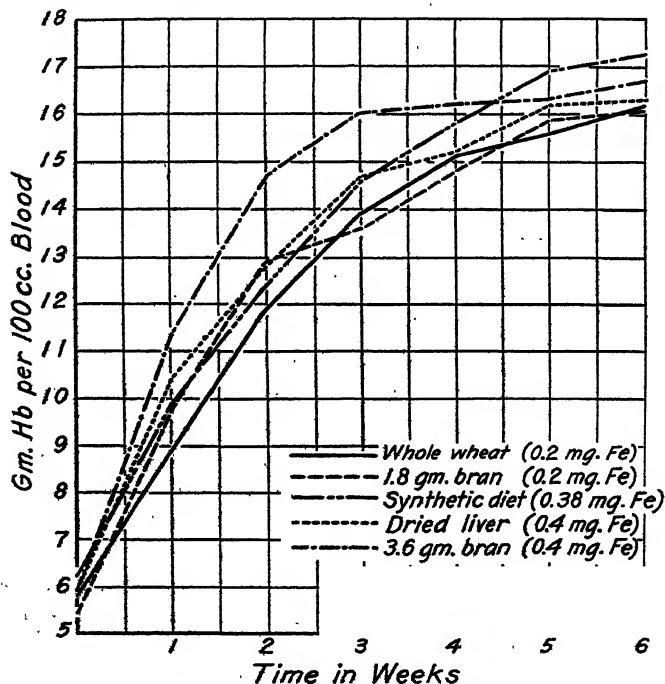


FIG. 1. Average weekly increases of hemoglobin of rats during feeding-up period.

1.8 gm. is in harmony with data of Mitchell and Miller (17) for a large number of animals showing a slightly more rapid rise with 0.5 mg. of iron daily than with 0.25 mg. Yet the animals on Diet K with an iron intake nearly the same as those on 3.6 gm. of bran (0.38 mg. and 0.40 mg. daily, respectively) had no more rapid regeneration than those on 1.8 gm. of bran or 6.5 gm. of whole wheat with only about half as much iron. The average increases

for the five groups for the 6 weeks period range between 10.4 and 11.2 gm. of hemoglobin per 100 cc. of blood, and the differences are too small to be considered significant.

The hemoglobin increase per 100 cc. of blood for the animals on 6.5 gm. of whole wheat agrees very closely with the findings of Rose and Vahlteich (1) and justifies averaging together the results of both investigations. For the total of twenty-five cases the average increase in 6 weeks is  $10.5 \pm 0.2$  gm. The differences between the increases on Diet K, which is known to be an optimal diet for the rat, and those on the milk diet plus the wheat or bran, do not indicate any less good utilization of the latter type of diet, so far as gains in hemoglobin per 100 cc. of blood are concerned.

While all the animals grew quite well, differences in rate of growth on the different diets were considerable. Increases seem to be closely correlated with the total calories consumed and to depend on the animals' willingness to eat the diet. The animals on Diet K ate more and gained more than any others, eight males increasing on the average 142 per cent of their weight and nine females, 106 per cent. The rats fed liver came second in food consumption and growth, the fourteen males increasing in weight 130 per cent and the five females, 86 per cent. Percentage increases in weight for all the groups are shown in Table II. In view of the fact that 3.6 gm. of bran, furnishing a liberal amount of iron daily and causing the most rapid regeneration of hemoglobin per unit volume of blood, did not stimulate the most rapid growth, it would seem that iron and other hemoglobin-building essentials can hardly be considered the controlling factors here. The food consumption for all groups calculated to calories per gm. of rat was fairly uniform, ranging from 0.27 to 0.31 calories, the highest figure being for rats on Diet K.

In order to take changes in body weight into account in judging results, total hemoglobin in the animal at different periods of the experiment has been calculated from tables derived by means of formulæ of Chisholm and Hatai, cited by Donaldson in his classic monograph on the rat (18). These tables give for each sex the weight of blood in gm. for different body weights. Fortunately, according to Scott and Barcroft (19) anemic and normal rats differ little in blood volume. Using the carbon monoxide method, they reported an average of 6.13 cc. of blood per 100 gm. of normal

rat and 6.65 cc. of blood per 100 gm. of anemic rat. In this study the total hemoglobin per rat has been estimated by converting blood weight to blood volume (taking specific gravity as 1.056 as given by Donaldson) and then multiplying the volume of blood for a given weight of animal by the amount of hemoglobin found per 100 cc. of blood. Although the increase of hemoglobin per unit volume of blood was almost identical for the five groups for the 6 weeks feeding-up period, the estimate of total increase in gm. per rat varied from an average of 1.19 gm. for those fed 1.8 gm. of bran to 1.72 gm. for those on Diet K.

The animals on 6.5 gm. of whole wheat and those on 3.6 gm. of bran were much alike as regards gain in weight and total gain of hemoglobin. Those on 1.8 gm. of bran made the lowest average gain in weight and consequently had the lowest total gain of hemoglobin. Only one male out of the nine on 1.8 gm. of bran exceeded 200 gm. at the end of the period, while only one out of fourteen on liver and one out of eight on Diet K did not exceed it. To rule out differences in size, the gm. of hemoglobin gained per gm. of gain in weight have also been estimated. The males of the different groups show more uniformity than the females and the latter in all cases give higher figures than the males, the differences between the sexes ranging from 19 per cent on bran at both levels and 21 per cent on Diet K to 30 per cent on liver and 64 per cent on whole wheat.

### *Bleeding Period*

During this period the hemoglobin content of the animals invariably decreased with successive bleedings. As it was found difficult to withdraw blood without fatality after reduction to about 6 gm. of hemoglobin per 100 cc. of blood, this was taken as the level to which all the animals should be reduced. Those which could not withstand more than two or three bleedings and succumbed before they reached the desired degree of anemia were discarded. It was observed that the blood of the anemic animals clotted more slowly, and many of the animals that died before reaching the desired low level had clots about the heart indicating failure of the cardiac puncture to be promptly occluded.

The final body weight without the digestive tract was arbitrarily adopted as the basis for converting iron of hemoglobin into

percentage of body weight (allowing 3.35 mg. of iron per gm. of hemoglobin). This was done for the sake of convenience in comparing these results with the analyses of the bodies for iron at the end of the bleeding period.

The length of time required to reduce the animals to the desired state of anemia was quite variable, due perhaps chiefly to the difficulty of withdrawing the same amount of blood each time. The average time and also the range for each group is given by sexes in Table III. There was a tendency for the time to be longer in case of females than males, but as this experiment was not specially planned for comparison of the sexes, more cases must be studied before any definite conclusion can be drawn. Throughout the bleeding period the animals remained practically stationary as to weight, the changes being mostly within  $\pm 20$  gm. Only one animal lost as much as 58 gm. and only one gained as much as 40 gm. The low food consumption and the gradually increasing anemia account for the failure to grow at an age when growth would ordinarily be continuing. The food intake per gm. of rat per day averaged from 0.20 to 0.27 cc. of milk. The iron intake approximated 0.0002 mg. of iron per gm. of rat per day. The milk as analyzed from time to time by Vahlteich (see Table I) averaged 0.08 mg. of iron per 100 cc. of milk, and this value was used in estimating the iron intake. This was not sufficient to cause any measurable regeneration between bleedings.

The hemoglobin removed has been calculated in terms of the weight of the rats at the beginning of the period but may be regarded as representing the whole time since the weight changes were comparatively small. Here again, differences between males and females overshadow differences between diets, amounts removed from the females being without exception higher than those from the males. Averages for each group by sexes are shown in Table III. Total iron removed is noticeably lower on 1.8 gm. of bran than on any other diet. This would seem to have furnished the minimum of iron for hemoglobin regeneration, and to have failed to supply something necessary for optimum growth (possibly not enough vitamin B). While the hemoglobin per 100 cc. of blood came up to normal, the animals were small, and in consequence less hemoglobin had to be removed to make them anemic.



Of twenty-two animals whose iron removed as hemoglobin was above 0.0026 per cent of the body weight, eighteen were females, and of these fourteen were above 0.0027, a figure not reached by any male. Considering findings with regard to calcium, it is not very surprising to find the females with a higher reserve than the males. Sherman and MacLeod (20) have shown that the calcium content of female rats which had not reared any young was higher than that of males. The females in this experiment, receiving the same amount of each iron-bearing supplement as the males, and gaining less in weight, had a better opportunity for storage, but that something more than this is involved is clear from the fact that males which did not gain as much in weight as the average for their sex did not store any more iron than those that grew more.

When 1.8 gm. of bran and 6.5 gm. of whole wheat, each yielding 0.2 mg. of iron, are compared, the total amount removed from the animals on whole wheat is distinctly greater, and so is the percentage of total intake, especially in case of the females.

Comparison of 1.8 gm. and 3.6 gm. of bran, the latter furnishing twice as much iron as the former, shows that 40 per cent more iron as hemoglobin was removed from the males and 70 per cent more from the females on 3.6 gm. As these animals grew more, the percentage of the total body weight, while greater, did not show such a striking difference, but the more rapid regeneration during the feeding-up period would seem to be correlated with the ability to yield hemoglobin during the bleeding period.

On comparing whole wheat with liver which furnished twice as much iron, there is found practically no difference in total amount, nor in per cent of body weight minus digestive tract. In other words, the liver was apparently only half as efficient as whole wheat in promoting hemoglobin formation.

When whole wheat was compared with 3.6 gm. of bran which furnished twice as much iron, the amount of hemoglobin removed was only about 20 per cent higher in the case of males and 10 per cent in the case of females, and as the rate of growth was similar, it would seem that the very efficiently used 0.2 mg. of iron from whole wheat furnished almost but not quite the maximum quantity of iron which the animals were capable of converting into hemoglobin under the conditions of the experiment.

Diet K, furnishing the same amount of iron as 3.6 gm. of bran,

yielded a slightly higher percentage of the intake as hemoglobin, but the average of the total iron removed was about the same as for the bran. This food should also be fed at the level of 0.2 mg. of iron, as it is evident that when the optimum amount for hemoglobin regeneration is reached, there is no advantage in a larger amount to be seen within the time limits of this experiment. The results with the animals fed Sherman's Diet 13, known to be ade-

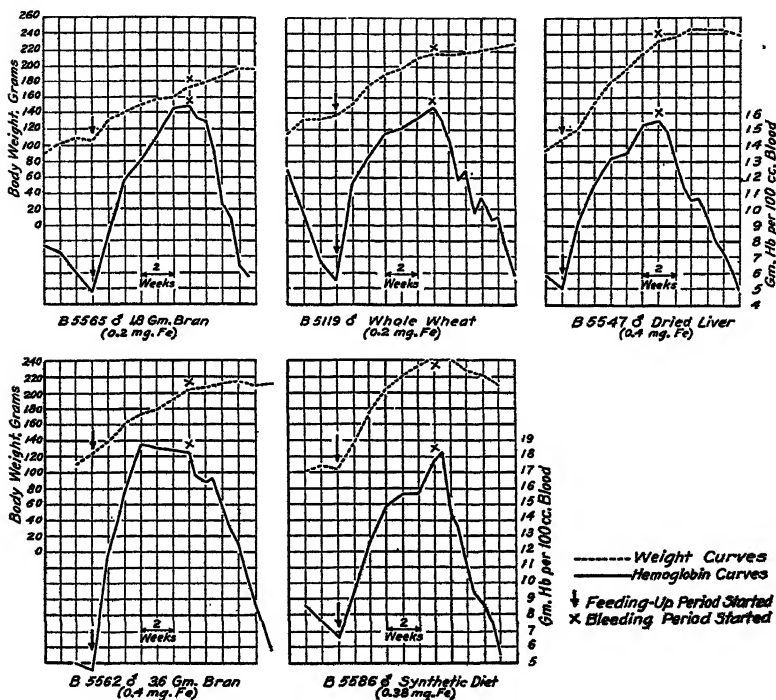


FIG. 2. Weekly changes in hemoglobin and body weight of rats during the entire experiment.

quate through many generations, are similar to those on 3.6 gm. of bran, and slightly higher than on 6.5 gm. of whole wheat, again indicating the efficiency with which 0.2 mg. of iron from whole wheat can be used by the rat, with a slight advantage from a little higher dosage. One typical case on each diet has been shown in Fig. 2, giving curves for weekly changes in hemoglobin and body weight.

*Iron Content of Bodies of Anemic Rats*

By reference to Table III, it will be noted that the averages for hemoglobin per 100 cc. of blood at the end of the bleeding period range between 5.5 and 6.1 gm. Of the 67 animals whose bodies were analyzed, 75 per cent were reduced to a level of between 5.5 and 6.1 gm., and the others ranged between 4.4 and 5.4 gm., mostly above 5.0 gm. The iron content of their bodies minus the digestive tract is shown in Table IV. The rats which grew least (those on 1.8 gm. of bran) had the smallest total amount of iron remaining in the body at death. The animals on Diet 13, which had endured no preliminary depletion period, and those fed liver, whole wheat, 3.6 gm. of bran, and Diet K grew at about the same rate and the total iron content of their bodies was about the same.

The iron content of the bodies expressed in percentage of weight minus the digestive tract is practically uniform for all depleted animals regardless of diet or sex. Even the controls, put on milk only at first at the age of 4 weeks and killed at the same time as the animals on the experimental diets, had the same amount of iron in their bodies. With the exception of seven animals the analyses of the 78 anemic rats showed values between 0.0016 and 0.0026 per cent and the average for all bodies analyzed is 0.0021 per cent. Considering the differences in dietary history, the inevitable individual variations, and the difficulties in analyzing rat bodies for the very small amounts of iron present, the results are remarkably constant.

Data on the iron content of anemic rats are rather scarce in the literature. Williamson and Ets (21) have reported analyses by the Ripper-Schwarzer method of bodies of rats fed the Osborne and Mendel casein diet with no modification except the withdrawal of ferric lactate from the salt mixture. This diet yielded them 0.072 mg. of iron per 100 gm. of food, but Diet K, in which the precaution was taken to remove much of the iron of yeast by making an alcoholic extract, still yielded 1.5 mg. of iron per 100 gm. of food. The animals of Williamson and Ets did not become anemic, as shown by hemoglobin tests, and their rat bodies had averages of 0.00755 per cent and 0.00595 per cent for two groups of animals on the so called low iron diet. These figures are higher than those of Smythe and Miller (11) on normal rats fed commercial calf meal which averaged 0.0051 per cent. Seven normal

males in this experiment show an average, 0.0043 per cent, identical with that shown by seven males of about the same age (90 to 120 days) reported by Smythe and Miller.

#### SUMMARY

Young rats from mothers on a normal diet were reduced to a practically uniform state of anemia by exclusive milk feeding, begun at the age of 28 days. When the blood hemoglobin dropped below 7 gm. per 100 cc. of blood, the animals were put on experimental diets found by preliminary trials to be adequate for return to the normal level of hemoglobin (16 gm. per 100 cc. of blood) in 6 weeks. Four of the diets consisted of milk supplemented daily as follows: (1) with 6.5 gm. of whole wheat yielding 0.2 mg. of iron; (2) with 1.8 gm. of prepared bran also yielding 0.2 mg. of iron; (3) with 1.6 gm. of dried liver yielding 0.4 mg. of iron; (4) with 3.6 gm. of prepared bran also yielding 0.4 mg. of iron. A fifth ration, called Diet K, yielding 0.38 mg. of iron daily, was an Osborne and Mendel diet, modified to reduce the iron by using an alcoholic extract of yeast, omitting ferric citrate from the salt mixture, and supplementing six times per week with 0.22 mg. of iron as ferric citrate.

During the feeding-up period there were no significant differences in the amounts of hemoglobin produced in terms of blood volume, as shown in Table II. The rate of regeneration is shown in Fig. 1. Animals fed milk plus 3.6 gm. of bran attained the normal level in 3 weeks, while the others required 5 or 6 weeks. Greatest growth occurred on liver and Diet K, next on whole wheat, and least on bran, and gains were closely correlated with total calories consumed rather than iron content. The average total hemoglobin production in 6 weeks per gm. of rat ranged from lowest to highest on the various supplements in the following order: 1.8 gm. of bran, 6.5 gm. of whole wheat, 3.6 gm. of bran, 1.6 gm. of dried liver, and Diet K. Normal regeneration did not occur on 0.8 gm. of dried liver. Hemoglobin gain per gm. of rat was from 20 to 64 per cent higher for females than males.

At the end of the 6 weeks feeding-up period, the animals were all depleted by repeated bleeding from the heart, until the hemoglobin was reduced to an average of about 6 gm. per 100 cc. of blood (range 4.4 to 6.2 gm.). To prevent hemoglobin regenera-

tion and maintain a fairly constant weight, the animals were all kept on an exclusive milk diet during the bleeding period. It was found that differences between sexes were marked, females per unit of weight yielding more hemoglobin than males, and thus indicating greater capacity for storage. Taking hemoglobin withdrawn from the animals on 0.2 mg. of iron in the form of whole wheat as the basis for comparison, those on 1.8 gm. of bran grew less well and produced slightly less hemoglobin; those on 0.4 mg. of iron as liver grew slightly better but produced no more hemoglobin. Animals on 0.4 mg. of iron as bran produced slightly more than those on 0.2 gm. of whole wheat, indicating that 0.2 mg. of iron is slightly below the optimum daily amount for the rat. Experience with other diets yielding more than the minimum requirement of iron in readily available form (Diet K, Diet 13) indicates that iron furnished in amounts above this optimum for maintenance of normal hemoglobin is not held in reserve.

This is confirmed by the findings regarding the iron content of the bodies at the end of the bleeding period, when the rats which had been reduced to a common state of anemia were chloroformed and their bodies minus digestive tracts analyzed for iron. There was a striking constancy regardless of diet or sex, the percentage for 78 bodies averaging  $0.0021 \pm 0.00003$  per cent. The tendency of females to store more iron than males is quite marked and will be subjected to further investigation. From 20 to 80 per cent more hemoglobin per gm. of rat had to be removed to induce the same degree of anemia.

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## THE RELATION OF THE DICARBOXYLIC AMINO ACIDS TO NUTRITION\*

BY R. REDER ST. JULIAN† AND WILLIAM C. ROSE

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

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The literature affords comparatively little information concerning the nutritive importance of the dicarboxylic amino acids. The present status of the problem has been reviewed elsewhere (Bunney and Rose, 1928; Rose, 1932), and need not be discussed in detail here. Suffice it to say that the limited data available point, for the most part, to the dispensable nature of these dietary components. This is surprising in view of the large proportions in which the dibasic acids, particularly glutamic acid, occur in the ordinary proteins of the diet. Teleologically, one might expect materials which are ingested under normal conditions in such abundance to play an unusually important rôle in human and animal economy. Indeed, this seems the more probable when one recalls that glutamic acid alone accounts for more than one-tenth of the entire weight of many tissue proteins, is a component of glutathione (Hopkins, 1929), and in the form of its amide, glutamine, serves as the detoxicating agent in man for phenylacetic acid (Shiple and Sherwin, 1922). With these considerations in mind, we have undertaken a reinvestigation of the relation of the dicarboxylic amino acids to growth.

### EXPERIMENTAL

In the removal of aspartic, glutamic, and hydroxyglutamic acids from casein the method of Kingston and Schryver (1924) was

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† The experimental data in this paper are taken from a thesis submitted by R. Reder St. Julian in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.



used. For this purpose, 3 kilos of casein were hydrolyzed with sulfuric acid according to the usual procedure employed in this laboratory (*cf.* Berg and Rose, 1929). After precipitation of the sulfuric acid with barium hydroxide, and repeated washing of the barium sulfate precipitate, the combined filtrate and washings were concentrated *in vacuo* to incipient crystallization. The solution was then treated with barium hydroxide in 30 per cent excess, as determined by a Sørensen titration, and the whole was added gradually with vigorous stirring to 3 volumes of 95 per cent alcohol.

TABLE I  
*Composition of Diet 1\**

Hydrolyzed casein from which dibasic amino acids had been precipitated.....	8.4
Tryptophane.....	0.2
Cystine.....	0.4
Dextrin.....	46.0
Sucrose.....	15.0
Salt mixture†.....	4.0
Agar.....	2.0
Lard.....	19.0
Cod liver oil.....	5.0
Total.....	100.0

\* Vitamin B factors were supplied in the form of pills containing 200 mg. of yeast daily. The control animals were given each day 130 mg. of glutamic acid and 50 mg. of aspartic acid incorporated in the vitamin pills.

† Osborne and Mendel (1919).

The above mixture was allowed to stand on ice for 4 days in order to secure complete precipitation. The barium salts of the dicarboxylic acids were then removed by filtration, and the filtrate was carefully freed of excess barium hydroxide in the usual manner. The solution was concentrated to a small volume *in vacuo*, transferred to evaporating dishes, and the drying completed, first on steam cones, and finally in a vacuum oven. The resulting material, amounting to 1092 gm., was thoroughly ground and passed through a 30 mesh sieve.

It will be observed that only about 36 per cent of the original casein was recovered in the final product. Had the dibasic acids

alone been removed, and the remaining amino acids quantitatively recovered, approximately 1900 gm. of material would have been secured. Evidently, the large loss is to be attributed in part to adsorption of other amino acids upon the heavy barium sulfate precipitates. But the greatest loss doubtless occurred during the precipitation of the dibasic acids from the very concentrated solution. We deliberately carried the evaporation of the hydrolysate to incipient crystallization in order to insure as complete precipitation as possible of the three amino acids in question. We expected that these conditions would lead to considerable loss of the less soluble amino acids. Under the circumstances, we are confident that the resulting material was as near devoid of aspartic, glutamic, and hydroxyglutamic acids as one can obtain by the Kingston and Schryver procedure.

The above mixture of amino acids, supplemented with suitable quantities of tryptophane and cystine, served as the sole source of nitrogen in the basal diet. The composition of the latter is shown in Table I. It will be observed that the nitrogenous components represent only 9 per cent of the ration. This low level was employed because of the experience of Osborne and Mendel (1915) that as the protein portion of the ration is diminished, the essential amino acid present in smallest amount may become the limiting factor in growth. By adopting the procedure of these authors we decreased the intake of any traces of the dibasic amino acids which may have failed to be precipitated.

Young rats served as the experimental animals. In addition to the basal diet, which was ingested *ad libitum*, the vitamin B factors were supplied to each rat daily in the form of a pill containing 200 mg. of yeast. The control animals were given each day 130 mg. of glutamic acid and 50 mg. of aspartic acid incorporated in the vitamin pills.

The experiments involved the use of several litters. In no case was the growth of the control animals, which received aspartic and glutamic acids, better than that of their litter mates without the supplements. The results obtained with a single litter are reproduced in Chart I. It is evident that all of the rats made remarkably rapid gains despite the low nitrogen content of the diet. In Table II are shown the figures representing the average daily gain in weight and the average daily food consumption of each

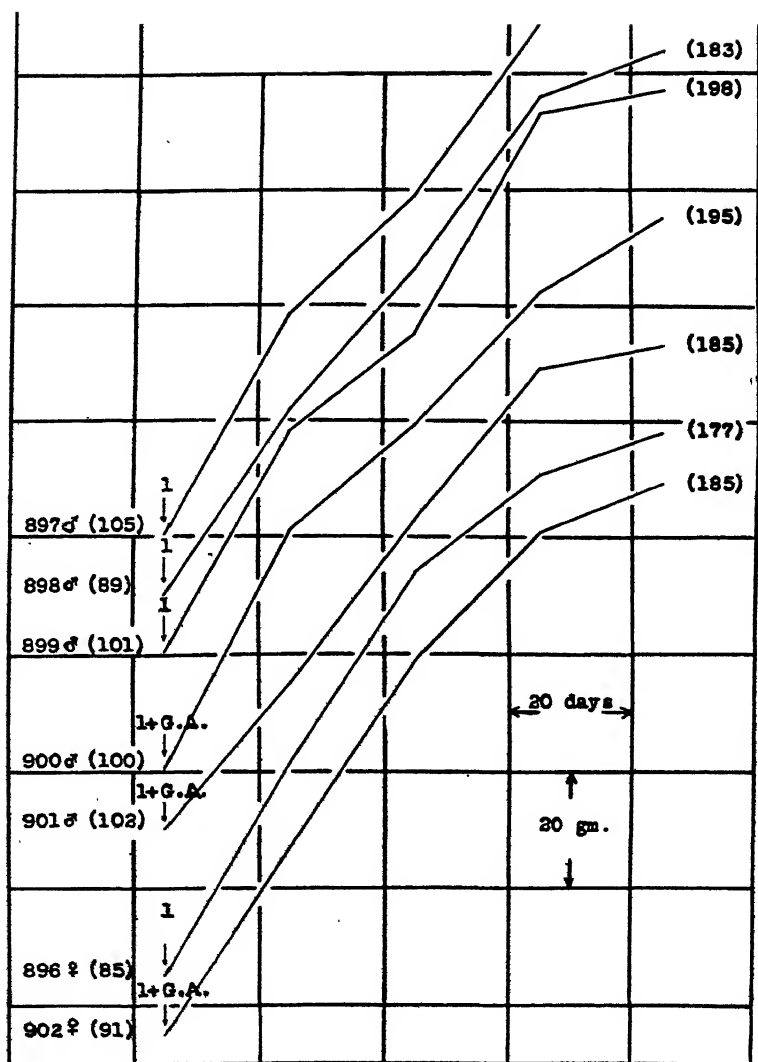


CHART I. The numbers in parentheses signify the initial and final weights of the rats. The arrows show the points at which the diets were begun, while the numbers just above the arrows correspond to the diet number. When the supplementing amino acids were fed, this fact is indicated by symbols following the diet number. The letters *G.A.* denote 130 mg. of glutamic acid and 50 mg. of aspartic acid administered daily. Thus 1 + *G.A.* signifies that Diet 1 was supplemented with the above amounts of glutamic and aspartic acids. The latter were incorporated in the vitamin pills.

animal. The data are strikingly uniform, and clearly demonstrate that the removal of the dicarboxylic amino acids by the procedure employed fails to alter the nutritive value of the resulting material.

TABLE II

*Food Consumption and Daily Gain in Body Weight of Experimental Animals*

Rat No. and sex	Diet No.	Average daily gain in body weight	Average daily food consumption
		gm.	gm.
897, ♂	1	1.2	7.3
898, ♂	1	1.3	7.6
899, ♂	1	1.2	6.9
900, ♂	1 + glutamic and aspartic acids	1.2	6.9
901, ♂	1 + glutamic and aspartic acids	1.0	7.2
896, ♀	1	1.1	7.9
902, ♀	1 + glutamic and aspartic acids	1.2	9.0

## SUMMARY

The removal of aspartic, glutamic, and hydroxyglutamic acids from hydrolyzed casein fails to diminish the nutritive value of the resulting material. Apparently the dicarboxylic amino acids are not indispensable dietary components.

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## PROLINE AND HYDROXYPROLINE IN NUTRITION\*

By R. REDER St. JULIAN† AND WILLIAM C. ROSE

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

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Of the twenty amino acids generally recognized as components of proteins only proline manifests the property of being appreciably soluble in absolute ethyl alcohol. According to Kapfhammer and Eck (1927), 100 gm. of a saturated solution of proline in absolute alcohol at 19° contains 1.5 gm. of the amino acid. At 40° the solubility is approximately 2.8 per cent (172.6 mg. in 6 cc. of alcohol). Furthermore, the same authors call attention to the fact that a solution of proline in absolute alcohol dissolves hydroxyproline. Thus 6 cc. of the solvent saturated with proline at 40° are said to dissolve 9.6 mg. of hydroxyproline.

The above observations justify the assumption that proline may be completely removed from a hydrolyzed protein by a sufficiently large number of extractions with absolute ethyl alcohol. How successfully hydroxyproline can be separated by this method is uncertain. So long as proline is being extracted, hydroxyproline would dissolve in the solvent. In the case of casein, which is believed to have a very low hydroxyproline content (0.23 per cent; Foreman, 1919), and a moderately high proline content (Dakin, 1918), there seems little reason to doubt that the former would be satisfactorily removed during the extraction of the latter. With edestin, however, which according to Abderhalden (1902-03) contains 2 per cent of hydroxyproline, the completeness of extraction would be open to question.

\* This communication was presented before the meeting of the American Society of Biological Chemists at Philadelphia, April, 1932.

† The experimental data in this paper are taken from a thesis submitted by R. Reder St. Julian in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

Inasmuch as the extraction method appears to be the best available for the removal of proline and at least part of the hydroxyproline, it was employed for the preparation of diets deficient in these amino acids. In so doing, we are fully aware of the possible limitations of the procedure. On the other hand, the extracted hydrolyzed proteins certainly contained greatly diminished amounts of the alcohol-soluble amino acids. Evidence for this fact will be presented below.

For a review of the inadequate and conflicting data already recorded in the literature concerning the nutritive importance of proline and hydroxyproline, the reader is referred to a recent paper by the senior author (Rose, 1932).

TABLE I  
*Proline and Hydroxyproline Present in Proteins Employed*

	Proline	Hydroxyproline
	<i>per cent</i>	<i>per cent</i>
Casein.....	7.63	0.23
Lactalbumin.....	3.76	?
Edestin.....	4.10	2.00

#### EXPERIMENTAL

Three proteins, namely casein, lactalbumin, and edestin, were made use of in the preparation of the proline-deficient diets. The casein was a commercial product. The lactalbumin and edestin were prepared in this laboratory from skim milk and hemp seeds respectively. The lactalbumin may have contained traces of casein. The proportions of proline and hydroxyproline said to be present in the proteins in question are reproduced in Table I. We know of no value for the hydroxyproline content of lactalbumin. Most analyses of edestin represent the amount of this amino acid with a question mark. The value given in Table I is that reported by Abderhalden (1902-03).

The proteins were hydrolyzed with sulfuric acid in the usual manner (Berg and Rose, 1929). After careful precipitation of the sulfuric acid with barium hydroxide, and thorough washing of the barium sulfate, the combined filtrate and washings from each protein were evaporated to dryness, first by means of a vacuum

still, and finally in a vacuum oven. Each of the resulting materials was then subjected to forty extractions with absolute ethyl alcohol. The proportions of the latter varied somewhat with the quantities of proline and hydroxyproline present in the proteins. Thus for each kilo of original casein 1 liter of the solvent was used in each extraction. With lactalbumin, having a lower proline content, about 950 cc. of alcohol per kilo were employed each time. In order to afford a better opportunity for the removal of hydroxyproline from edestin, each extraction involved the use of approximately 1300 cc. of the solvent per kilo of the protein.

In making the extractions, the finely ground hydrolyzed protein was suspended in alcohol, and the mixture was heated to boiling, with frequent shaking, on a steam cone. The solution was then cooled in an ice bath to about 10°, and immediately filtered. This process was repeated forty times. The alcoholic filtrates were combined into groups of five, Filtrates 1 to 5, 6 to 10, 11 to 15, etc., and evaporated to dryness. The non-amino nitrogen was then determined in each of the eight fractions obtained from each protein in order to secure a rough measure of the rapidity of removal of the soluble material. These data are not reproduced in full inasmuch as they cannot be taken as accurate estimates of proline and hydroxyproline, since traces of other forms of non-amino nitrogen (arginine and histidine?) doubtless dissolved in the alcohol. However, it is significant that with each protein the first three fractions (fifteen extracts) carried most of the non-amino nitrogen, while the last five fractions showed small, approximately constant amounts. This may be illustrated with casein. The first three fractions from 1 kilo of the protein showed 6.6, 2.4, and 1.3 gm. of non-amino nitrogen respectively. The remaining five fractions varied from a maximum of 0.73 gm. to a minimum of 0.37 gm. of non-amino nitrogen. Similar figures were obtained with the lactalbumin and edestin extracts. It seems probable, therefore, that most of the proline, and possibly a large part of the hydroxyproline, had been removed in the first fifteen extracts. With each protein the non-amino nitrogen recovered from the forty extracts was considerably larger than that necessary to account for the proline and hydroxyproline present, the figures recorded in Table I being assumed correct.

Finally, the dried residues remaining after the extractions were





amounts were present because of the smaller quantities of the prolines; but in any event, the values tabulated probably are not too high. The data in Table II show, after the corrections indicated above are made, that the non-amino nitrogen unaccounted for amounted to approximately 0.5 per cent for casein, 0.6 per cent for lactalbumin, and 0.7 per cent for edestin. When one recalls that these figures possibly include traces of peptide nitrogen, and certainly some nitrogen in the form of diketopiperazines produced

TABLE III  
*Composition of Diets\**

	Diet 1	Diet 2	Diet 3
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Proline-free casein.....	8.4		
"    lactalbumin.....		8.6	
"    edestin.....			8.0
Tryptophane.....	0.2	0.2	0.2
Cystine.....	0.4	0.2	0.4
Lysine.....			0.4
Dextrin.....	46.0	42.0	42.0
Sucrose.....	15.0	15.0	15.0
Salt mixture†.....	4.0	4.0	4.0
Agar.....	2.0	2.0	2.0
Lard.....	19.0	23.0	23.0
Cod liver oil.....	5.0	5.0	5.0
	100.0	100.0	100.0

\* Vitamin B factors were supplied in the form of two pills daily each containing 50 mg. of Harris yeast extract. Each of the control rats received 50 mg. of proline daily admixed with the vitamin extract.

† Osborne and Mendel (1919).

by the repeated hot alcohol treatments, it appears that the quantities of proline (and hydroxyproline?) present must have been quite small.

By use of the materials prepared as indicated above, three diets were formulated, as represented in Table III. These were employed in the growth studies. Young white rats served as the experimental animals. The total nitrogenous components in the basal diets amounted to 9 per cent (*cf.* Osborne and Mendel, 1915). The hydrolyzed proteins were supplemented in each case with

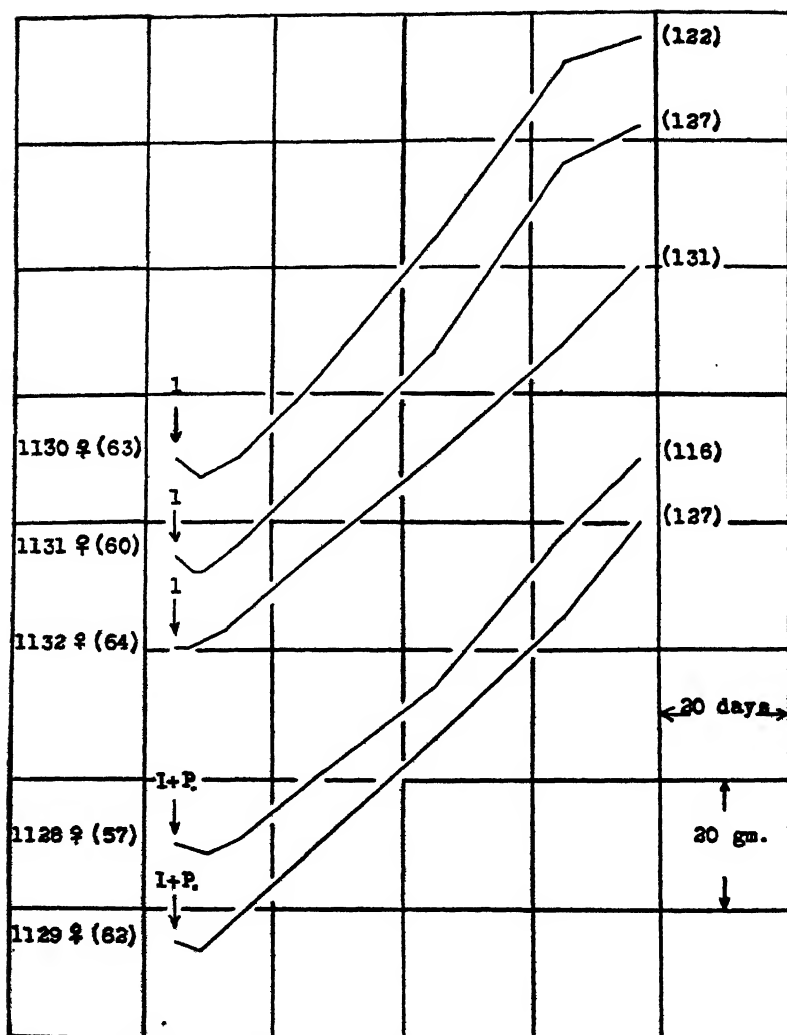


CHART I. Litter F. The numbers in parentheses signify the initial and final weights of the rats. The arrows show the points at which the diets were begun, while the numbers just above the arrows correspond to the diet numbers. When proline was fed, this fact is indicated by the symbol  $I + P$ . Thus  $I + P$  signifies that Diet 1 was supplemented with 50 mg. of proline daily. The supplement was incorporated in the vitamin pills.

cystine and tryptophane. Lysine also was added to the edestin ration (Diet 3) because of the low lysine content of this protein. Vitamin B factors were supplied in the form of two pills daily

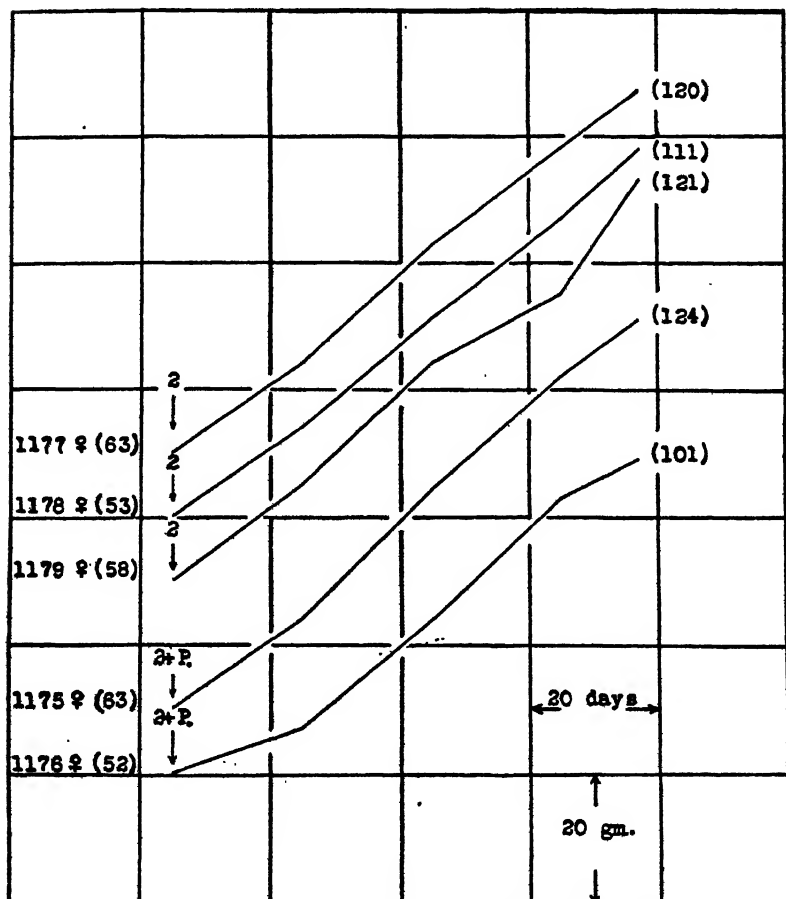


CHART II. Litter G. The figures and symbols have the same meaning as in Chart I.

each containing 50 mg. of Harris yeast extract. The control rats received 50 mg. of proline daily admixed with the vitamin extract. The basal diets were kept before the animals at all times.

The growth curves are reproduced in Charts I to III inclusive. Chart I shows the results with the casein hydrolysate. It is evident that the animals succeeded in growing at fairly rapid rates,

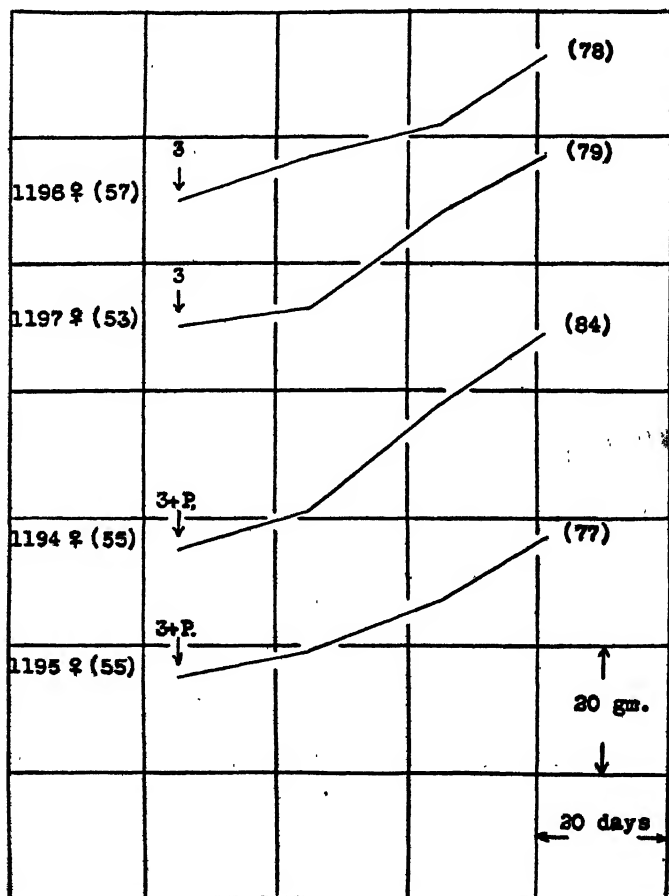


CHART III. Litter H. The figures and symbols have the same meaning as in Chart I.

and that those which received proline (Rats 1128 and 1129) did not make greater gains than did their litter mates upon the basal ration alone. Similar data were obtained in the lactalbumin (Chart II)

experiments. The animals upon the hydrolyzed edestin (Chart III) grew less satisfactorily than did those in the other two groups; but evidently the deficiency is not to be accounted for by the absence of proline inasmuch as the administration of the latter failed to exert a detectable improvement.

In Table IV are exhibited figures representing the average daily gain in weight and average daily food consumption of each animal. It is obvious that the variations in each group are well within the

TABLE IV

*Food Consumption and Daily Gain in Body Weight of Experimental Animals*

Rat No. and sex	Diet No.	Average daily gain in body weight	Average daily food consumption
		gm.	gm.
1130, ♀	1	0.81	5.7
1131, ♀	1	0.93	5.8
1132, ♀	1	0.93	6.1
1128, ♀	1 + proline	0.81	5.4
1129, ♀	1 + "	0.90	6.2
1177, ♀	2	0.79	6.2
1178, ♀	2	0.80	5.0
1179, ♀	2	0.85	5.6
1175, ♀	2 + proline	0.84	5.8
1176, ♀	2 + "	0.68	4.5
1196, ♀	3	0.37	4.2
1197, ♀	3	0.46	4.2
1194, ♀	3 + proline	0.51	5.4
1195, ♀	3 + "	0.33	4.1

limits of experimental error, and are not associated with the presence or absence of proline.

The above results appear to indicate that proline is not a necessary dietary constituent. We refrain from making a similar deduction concerning hydroxyproline since we are not certain as to the completeness of its removal by the procedure employed. Furthermore, the supplementing effect of hydroxyproline was not tested inasmuch as this amino acid was not available to us at the time these experiments were undertaken. Unquestionably, most of the hydroxyproline was extracted, at least from casein, by the forty alcohol treatments. Under the circumstances we are in-

clined to believe that this amino acid also is dispensable. After the above experiments had been completed, Adeline (1931) reported that rats upon diets containing 6 per cent of edestin cease growing after approximately 12 weeks, but that the addition of either proline or hydroxyproline remedies the nutritive deficiency and induces growth. The author concludes that the two compounds are interchangeable in metabolism, but that one must be present in the diet. Unfortunately, her data are not as clear-cut as one would wish. As will be discussed in the following paper, we have considered the possibility of several 5-carbon amino acids being interchangeable with proline, but have obtained no evidence for such a relationship. In our opinion, further and more convincing proof must be presented before the mutual interchangeability of proline and hydroxyproline can be accepted as an established fact. In the present paper we are particularly concerned with the fact that proline is a dispensable dietary constituent.

#### SUMMARY

The dry mixtures of amino acids obtained by the acid hydrolysis of casein, lactalbumin, and edestin, have been subjected to forty extractions each with absolute ethyl alcohol for the removal of proline and an unknown part of the hydroxyproline. The resulting materials, when incorporated in diets at 9 per cent levels (including suitable amino acid supplements) enable rats to grow at moderately rapid rates. The administration of 50 mg. of proline daily fails to accelerate the increase in body weight. It appears, therefore, that proline is a dispensable amino acid.

In view of the uncertainty as to the completeness of removal of hydroxyproline by the method employed, no positive statement can be made concerning its nutritive importance. We are inclined to the belief that it also is an unnecessary dietary component, but realize that final conclusions must await the results of further investigations.

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## THE POSSIBLE INTERCHANGEABILITY IN NUTRITION OF CERTAIN 5-CARBON AMINO ACIDS\*

By R. REDER ST. JULIAN† AND WILLIAM C. ROSE

(From the Laboratory of Physiological Chemistry, University of Illinois,  
Urbana)

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In view of the outcome of the preceding papers (St. Julian and Rose, 1932, *a*; 1932, *b*), pointing to the dispensable nature of the glutamic acids, proline, and possibly hydroxyproline, it occurred to us that these amino acids and the ornithine part of arginine might all be mutually interchangeable in metabolism. Publications from this laboratory (Bunney and Rose, 1928; Scull and Rose, 1930) have already indicated the dispensable nature of arginine. If the glutamic acids, the prolines, and ornithine were interchangeable, the absence from the diet of any four might not lead to nutritive failure until the fifth had been removed.

Such a relationship is suggested by the general similarity in chemical structure of the five compounds. Thus glutamic acid might lose a molecule of water with the formation of  $\alpha$ -pyrrolidone-carboxylic acid, a reaction accomplished *in vitro* by Abderhalden and Kautzsch (1910). The reduction of  $\alpha$ -pyrrolidonecarboxylic acid would result in the formation of proline; similarly, hydroxyglutamic acid might be a source of proline provided the  $\beta$ -hydroxyl group could undergo reduction. The loss of ammonia between the two amino groups of ornithine would lead to a direct production of proline. Furthermore, the above reactions might be reversible, and permit any one of the five compounds to assume vicariously

\* This communication was presented before the meeting of the American Society of Biological Chemists at Philadelphia, April, 1932.

† The experimental data in this paper are taken from a thesis submitted by R. Reder St. Julian in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

the functions of the others. In this connection the fact should be recalled that all of these acids are believed to share like catabolic fates in the completely diabetic organism, with the transformation into extra glucose of 3 of their carbon atoms.

With these considerations in mind we have prepared a casein hydrolysate as devoid as possible of the amino acids in question, and have employed it in feeding experiments as outlined below.

#### EXPERIMENTAL

4 kilos of casein were hydrolyzed and freed of the dibasic amino acids as described in the first paper of this series (St. Julian and Rose, 1932, *a*). In so doing, of course, aspartic acid as well as the glutamic acids was precipitated. This is of no consequence inasmuch as the absence from the food of aspartic acid does not lead to a decrease in the nutritive value of the remaining materials. After removal of the excess barium hydroxide and alcohol, arginine and histidine were precipitated as their silver salts according to the method of Vickery and Leavenworth (1927). These salts were filtered off, and the filtrate was freed of excess silver and barium hydroxide in the usual manner. The final filtrate was concentrated to a small volume *in vacuo*, and dried in a vacuum oven.

The dried amino acid mixture, amounting to about 1400 gm., was then subjected to forty extractions with absolute alcohol for the removal of proline, and at least a large part of the hydroxyproline. The method employed was identical with that already described (St. Julian and Rose, 1932, *b*) except that the proportions of alcohol were slightly different. For the first extraction 4 liters were employed. For the second to twelfth extractions, 2 liters were used each time. Each of the last twenty-eight extractions involved the use of 1.3 liters. Thus a total of approximately 62 liters of absolute alcohol was employed in an effort to remove the prolines completely.

Analyses showed that the final product contained only 0.69 per cent of non-amino nitrogen. If one corrects for 5 per cent of the lysine nitrogen, as explained in the preceding paper (St. Julian and Rose, 1932, *b*), the non-amino nitrogen is further reduced to 0.62 per cent.

Young rats served as the experimental animals. The composition of the diets is presented in Table I. Diet 1, which was

supplemented with suitable quantities of tryptophane, cystine, and histidine, contained 10.4 per cent of the hydrolysate so as to provide the nitrogenous materials at a level of 11.5 per cent. The latter was employed, instead of a 9 per cent level as in the preceding papers, because of the large proportion of the hydrolyzed casein which had been removed by the various steps in the process, thereby affording opportunity for the occlusion or adsorption of

TABLE I  
*Composition of Diets\**

	Diet 1	Diet 2
	<i>per cent</i>	<i>per cent</i>
Hydrolyzed casein from which eight amino acids had been removed.....	10.4	8.0
Tryptophane.....	0.2	0.2
Cystine.....	0.4	0.4
Histidine.....	0.5	0.5
Arginine.....		0.6
Aspartic acid.....		0.2
Glutamic ".....		1.0
Proline.....		0.6
Dextrin.....	43.5	43.5
Sucrose.....	15.0	15.0
Salt mixture†.....	4.0	4.0
Agar.....	2.0	2.0
Lard.....	19.0	19.0
Cod liver oil.....	5.0	5.0
	100.0	100.0

\* Vitamin B factors were supplied in the form of two pills daily, each containing 50 mg. of Harris yeast extract.

† Osborne and Mendel (1919).

some essential protein component. Diet 2 was the control ration. In addition to tryptophane, cystine, and histidine, it was supplemented also with arginine, aspartic acid, glutamic acid, and proline. This necessitated a reduction of the hydrolysate to 8 per cent in order to maintain the total nitrogenous materials at the same level used in Diet 1. With each animal, the vitamin B factors were fed separately in the form of two pills daily each containing 50 mg. of Harris yeast extract.

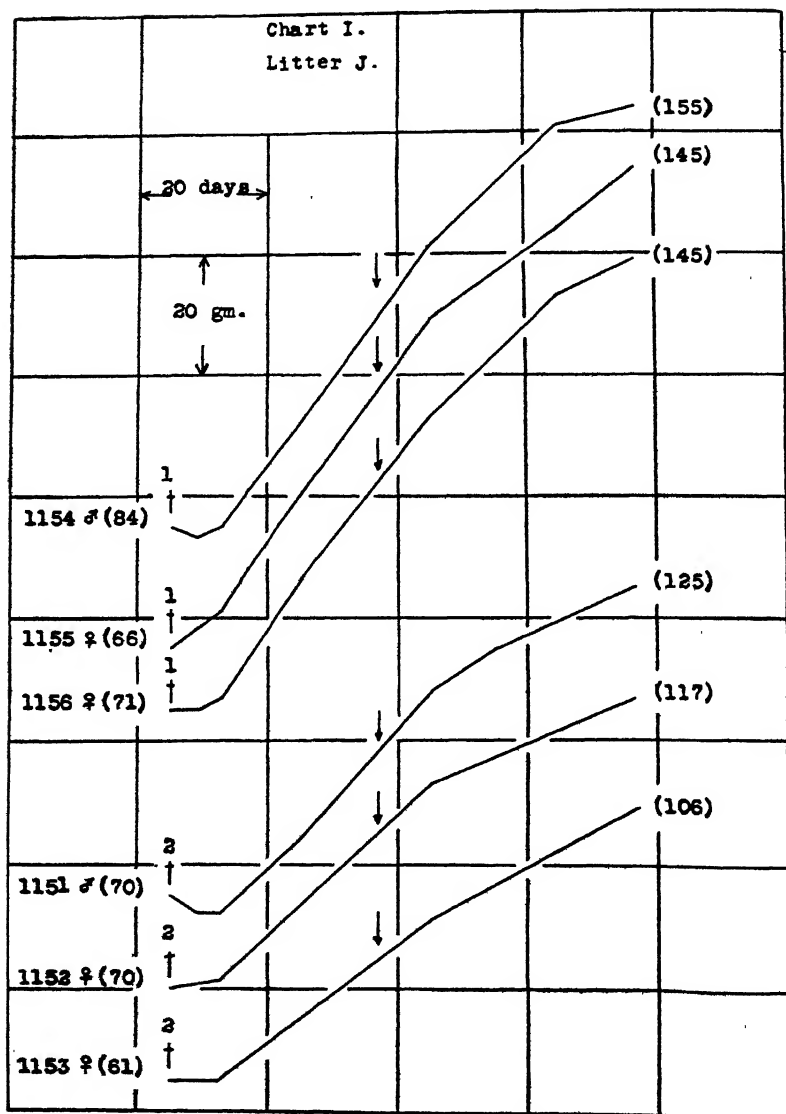


CHART I. The numbers in parentheses signify the initial and final weights of the rats. The daggers show the points at which the diets were begun, while the numbers just above the daggers correspond to the diet numbers. The arrows denote the points at which the reprecipitated hydrolysate was introduced into the rations.

The growth of three rats upon each of the two diets is graphically represented in Chart I. In Table II are recorded the average daily gain in weight, and the average daily food intake of each animal. It will be observed that the rats upon Diet 1, without the 5-carbon amino acids, made somewhat more rapid gains than did their litter mates upon Diet 2. This indicates that the presence of arginine, aspartic acid, glutamic acid, and proline did not improve the nutritive quality of the food. Probably the explanation for the slower growth of the rats upon Diet 2 is to be found in the reduction of the hydrolysate from 10.4 to 8 per cent, as explained above, so that some essential amino acid became the limiting factor. However, this does not alter the fact that the animals upon Diet 1, carrying only 10.4 per cent of a casein hydrolysate,

TABLE II  
*Food Consumption and Daily Gain in Weight of Experimental Animals*

Rat No. and sex	Diet No.	Average daily gain in body weight	Average daily food consumption
		gm.	gm.
1154, ♂	1	0.98	7.6
1155, ♀	1	1.09	6.9
1156, ♀	1	1.02	7.4
1151, ♂	2	0.69	8.0
1152, ♀	2	0.65	7.3
1153, ♀	2	0.62	6.8

were capable of making gains of approximately 1 gm. per day. This is the more remarkable when one recalls that the six amino acids absent from the ration account for almost 50 per cent of the entire casein molecule.

After these feeding experiments had gotten under way, the results of other investigations in this laboratory indicated that arginine may be precipitated more completely by the flavianic acid method of Kossel and Gross (1924) than by the silver procedure. Therefore, in an attempt to reduce further the arginine content of Diet 1, 320 gm. of the hydrolysate from which the several amino acids had been precipitated, were dissolved in 2600 cc. of distilled water, made acid to Congo red by the addition of sulfuric acid, and treated under constant stirring with 100 gm. of flavianic acid dissolved in 50 cc. of boiling water. The solution

was allowed to stand on ice for 4 days, after which the precipitate was filtered off. The filtrate was then treated with barium hydroxide for the removal of the excess flavianic and sulfuric acids, filtered, and the precipitate thoroughly washed. The reaction of the combined filtrate and washings was then carefully adjusted until neither the barium nor sulfate ion could be detected. After again being filtered, the solution was evaporated to dryness *in vacuo*. 288 gm. of amino acids were recovered.

The reprecipitated material was introduced into the diets at the points indicated by the arrows (Chart I). As will be observed, only a slight decrease in growth rate occurred. Furthermore, the effect was just as pronounced with the control rats (Diet 2) which received the supplements of arginine, dibasic acids, and proline, as in the group without these supplements (Diet 1). Thus, if the slightly slower growth rate is significant at all, it is not related to the 5-carbon amino acids with which we are concerned in this paper.

In view of the above results, it appears that arginine, the dibasic amino acids, and the prolines may all be removed from hydrolyzed casein without altering the nutritive value of the remaining materials. Contrary to our expectations, no evidence for an interchangeable relationship was secured. While we are inclined to the belief that all of these protein components are dispensable dietary constituents, we are fully aware that the methods employed, though the best available at the present time, may not have accomplished a quantitative removal of the acids in question. Obviously, final conclusions must await the results of experiments in which mixtures of highly purified amino acids entirely replace the protein part of the diets. The successful use of such mixtures will depend upon the isolation of the unknown growth essential, the existence of which has previously been demonstrated (Windus, Catherwood, and Rose, 1931-32), or the preparation of a concentrate of this unknown substance uncontaminated with the 5-carbon acids. Already, progress in both directions has been made in this laboratory, and will be reported later.

#### SUMMARY

Arginine, the dibasic amino acids, and the prolines, have been removed from hydrolyzed casein, and the remaining amino acids

employed as the sole source of nitrogen (except that present in 100 mg. of yeast extract daily) in otherwise adequate diets. Rats receiving such food mixtures grow at about half the normal rate when the hydrolysate, including supplements of tryptophane, cystine, and histidine, is supplied at a level of 11.5 per cent. Incorporating suitable quantities of arginine, aspartic acid, glutamic acid, and proline, in place of equivalent amounts of the hydrolysate, fails to improve the nutritive quality of the food.

The above results appear to indicate that the 5-carbon amino acids, ornithine, glutamic acid, hydroxyglutamic acid, proline, and hydroxyproline, are not capable of playing an interchangeable rôle in nutrition. However, final judgment must await the results of feeding experiments involving the use of mixtures of highly purified amino acids known to be entirely devoid of the compounds in question.

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# THE METABOLISM OF CYSTINE AND METHIONINE

## THE AVAILABILITY OF METHIONINE IN SUPPLEMENTING A DIET DEFICIENT IN CYSTINE\*

By RICHARD W. JACKSON† AND RICHARD J. BLOCK

(From the Laboratory of Physiological Chemistry, Yale University, New Haven)

(Received for publication, August 1, 1932)

Preformed protein, or its hydrolysis products, is one of the inevitable needs of the more complexly organized animal body. The particular metabolic requirement for each individual amino acid yielded by protein is, therefore, a matter of outstanding importance. Although it has been clearly recognized for some 20 years that amino acids might well differ in their degrees of nutritional significance, knowledge in this field is still incomplete.<sup>1</sup> The literature affords only a few examples of protein units which have been repeatedly demonstrated to be essential. That the animal's synthetic powers with respect to even these substances are not *absolutely* limited is shown by the fact that in specific instances they may be replaced in the diet by the corresponding lactic and pyruvic acids. The apparent dispensability of one amino acid may, moreover, depend on the capacity of another or others to play the rôle of substitute. Such has been thought to be the case with glycine. Metabolic interchange of this type may well explain some of the difficulty encountered in attempts to formulate diets

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† National Research Council Fellow in Medicine, 1930-31.

<sup>1</sup> Rose (1931-32) has presented a critical review of the amino acid literature giving the current status of the best facts with regard to the essential or non-essential nature of the known amino acids. Rose and his associates have shown that the known amino acids including methionine are not sufficient to support growth; and that at least one other indispensable factor, apparently an amino acid, is yet to be discovered.

deficient in protein components of more intricate constitution. In fact, there have been reported several studies designed to test such a conceivable physiological connection between amino acids of obviously similar chemical structure, as for example, between tyrosine and phenylalanine.

Cystine (with cysteine) long enjoyed a particular significance by virtue of its being the only naturally occurring amino acid known to contain sulfur and the only apparent major source of reduced sulfur for the body. The discovery of methionine by Mueller in 1923, however, introduced a new aspect to the situation. Methionine, like other amino acids derived from protein, is catabolized in the animal body (Mueller, 1923-24), but there has hitherto not been any basis for predicting a common metabolic behavior for the two sulfur-containing substances. In fact, inspection of the two formulas (cystine,  $(-\text{S}\cdot\text{CH}_2\cdot\text{CH}\cdot\text{NH}_2\cdot\text{COOH})_2$ , and methionine,  $\text{CH}_3\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}\cdot\text{NH}_2\cdot\text{COOH}$ ) reveals a lack of striking structural similarity. However, in view of the emphasis which many writers have placed on the presence of sulfur in cystine, the mere fact that methionine is the only known additional sulfur-bearing amino acid thus far isolated from proteins suggests the importance of ascertaining its physiological rôle experimentally. We are not familiar with any previous investigation of the metabolic interrelation of cystine and methionine.<sup>2</sup> These considerations led us to study the effect of administering methionine to animals restricted in growth through the limitation of the cystine intake.

#### EXPERIMENTAL

Male rats were employed in this study. When the animals reached a body weight of 60 to 75 gm. each, they were confined singly in cages equipped with false bottoms and the ration was changed from the stock food mixture to one deficient in cystine.

\* Sherman and Woods (1925) in commenting upon their biological method for the assay of cystine in casein refer to "cystine (or cystine plus nutritionally equivalent sulfur-containing radicles)." Again, Rimington and Bekker (1932) state, "Evans concludes, as have others, that there must be present in the diet [of sheep] some substance closely related to cystine from which the latter can be synthesized." Neither Sherman and Woods nor Rimington and Bekker, however, suggest a metabolic relationship between methionine and cystine.

*Basal Diet*—Different deficient diets fashioned after those of Osborne and Mendel (1915), Sherman and Merrill (1925), Lewis and Lewis (1926), and Rose and Huddlestun (1926) were tried with the purpose of securing a basal regimen which would within reasonable limits just maintain body weight. The lentil food described by Jones and Murphy (1924) was found to permit much too rapid growth. The formula of the basal diet (Diet B) finally selected was as follows: whole milk powder 15, gelatin 2, salt mixture (Osborne and Mendel, 1919) 1, sodium chloride 1.7, cornstarch 54.7, a dry Lloyd's reagent-vitamin B adsorbate preparation<sup>3</sup> 0.6, and lard 25 per cent. These ingredients were thoroughly worked into a doughy mixture which could be dispensed conveniently and quantitatively. This diet and its supplemented forms were, with one exception to be discussed later, fed *ad libitum*.

Each rat was supplied daily with a supplement of 100 mg. of cod liver oil and 125 mg. of dried yeast. There is no doubt that 100 mg. of an average cod liver oil furnishes an abundance of vitamins A and D for an albino rat. But there is some question as to whether the daily intake of whole milk powder, yeast, and vitamin B adsorbate would meet the animal's nutritional demands for vitamins B and G and other accessory water-soluble substances throughout an indefinitely prolonged experiment. However, vitiation of our experiments on this or similar account was precluded by the strict control standards imposed on every animal.

The supplementary feeding experiments were not undertaken until the animals on the cystine-poor diet had attained a condition of equilibrium with respect to body weight change. This usually occurred in the course of about 4 weeks. The chief difficulty encountered was that of too rapid growth; and for this reason approximately 50 per cent of all the animals originally placed upon the basal diet was discarded. Variations which different animals thus exhibit even when they are ingesting the same ration are believed to be due to inherent individual differences in appetite or food utilization efficiency. These factors are undoubtedly fairly constant, however, in a given animal restricted in growth in consequence of a constantly operating dietary amino acid deficiency. This point is borne out by the repeated observation that following

<sup>3</sup> This concentrate was kindly furnished by Eli Lilly and Company of Indianapolis.

an animal's initial adjustment to the diet, the food consumption and the body weight change are rather constant over long periods. Accordingly, animals with *fairly* stationary body weights were selected for the metabolism tests in order that any alteration in the growth rate would be convincingly manifested.

Every experiment was controlled by the behavior of the animal on the basal diet and on the basal diet supplemented with cystine or other growth-stimulating substance. Our experience led us to the conclusion that the general method outlined here, namely that of studying successive periods of feeding on the same animal rather than simply making comparisons of different animals, is the more reliable unless one resorts to the use of large numbers of animals and prolonged feeding periods. However, the growth of our test rats was at all times controlled by the behavior of other animals simultaneously subsisting on the basal diet alone. Thus various general laboratory factors were shown not significantly to affect the outcome of any experiment.

*Amino Acid Preparations*—The cystine, methionine, serine, alanine, aspartic acid, tryptophane, hydroxyproline, histidine monochloride, glycine, and phenylalanine specimens employed were colorless and characteristically crystalline. Their purity was established by nitrogen determinations. The cystine, aspartic acid, tryptophane, hydroxyproline, and histidine were all the natural optically active forms. The remaining amino acids were racemic preparations. The methionine was synthesized according to the directions of Windus and Marvel (1930).<sup>4</sup> These compounds prior to feeding were thoroughly mixed into the basal diet. 120 mg. were selected as the equivalent of cystine to be used as a supplement for 100 gm. of the basal diet. The other substances, unless otherwise specified, were employed in the amount of 2 or

<sup>4</sup> The methionine employed in this research was prepared collaboratively by H. M. Vars and R. W. Jackson, with the purpose of making methionine available for experiments in which they were respectively interested. The authors wish to express their appreciation to Dr. Vars for his cooperation in this arrangement which in a large measure made possible the metabolism experiments. Dr. Marvel, of the University of Illinois, kindly supplemented the original directions for this synthesis with certain modifications (to appear in the "Organic syntheses"). It may be stated that the method of Windus and Marvel proceeds smoothly and may be used to prepare any desired quantity of methionine from readily available starting materials.

4 molecular equivalents: 298 (2 equivalents) and 596 mg. (4 equivalents) of methionine, 420 mg. (4 equivalents) of serine, and 356 mg. (4 equivalents) of alanine, respectively, per 100 gm. of food.

#### DISCUSSION

The administration of methionine to animals subjected to the experimental regimen described above led to unmistakable increments in body weight. This is indicated in Charts I to IV. Attention is directed to the behavior of Rats 65 and 80 (Chart I) following the addition of methionine to the diet. The first of these gained 59 gm. in 7 weeks as contrasted to a previous body weight increase of 2 gm. in 4 weeks. The second, which was originally gradually losing weight, began to grow at quite an appreciable rate. Eight other animals exhibited similar responses to methionine. It may further be pointed out that animals growing in response to a dietary accession of methionine or cystine ceased to grow when deprived of these supplements, as illustrated in the growth curves for Rats 51 and 75 (Chart I). A summary of the effect of methionine ingestion upon the growth of a group of ten rats is presented in Chart IV. The average weight increment during the 2 weeks following methionine administration was 20 times greater than the mean increase preceding the inclusion of this amino acid in the diet.

It appears to be an established fact that methionine, like cystine, supplements the type of diet employed in these studies—a diet ordinarily termed cystine-deficient. This discovery originally reported in *Science* (Jackson and Block, 1931) has now been confirmed by Weichselbaum, Weichselbaum, and Stewart (1932). Their announcement states that the addition of methionine to the Sherman and Merrill (1925) diet fed throughout in constant daily portions caused approximate doubling of the growth rate of their rats. That the limitation of the food intake during the period of methionine administration did not prevent growth stimulation is in agreement with our results of a similar experiment discussed below.

*Dietary Specificity of Methionine and Cystine*—Inasmuch as the protein fraction of the basal diet was relatively small (6 to 7 per cent of the total weight), it was thought that perhaps the reaction to methionine might be due simply to an increase in the amount of any kind of usable nitrogen ingested at a given caloric intake. In

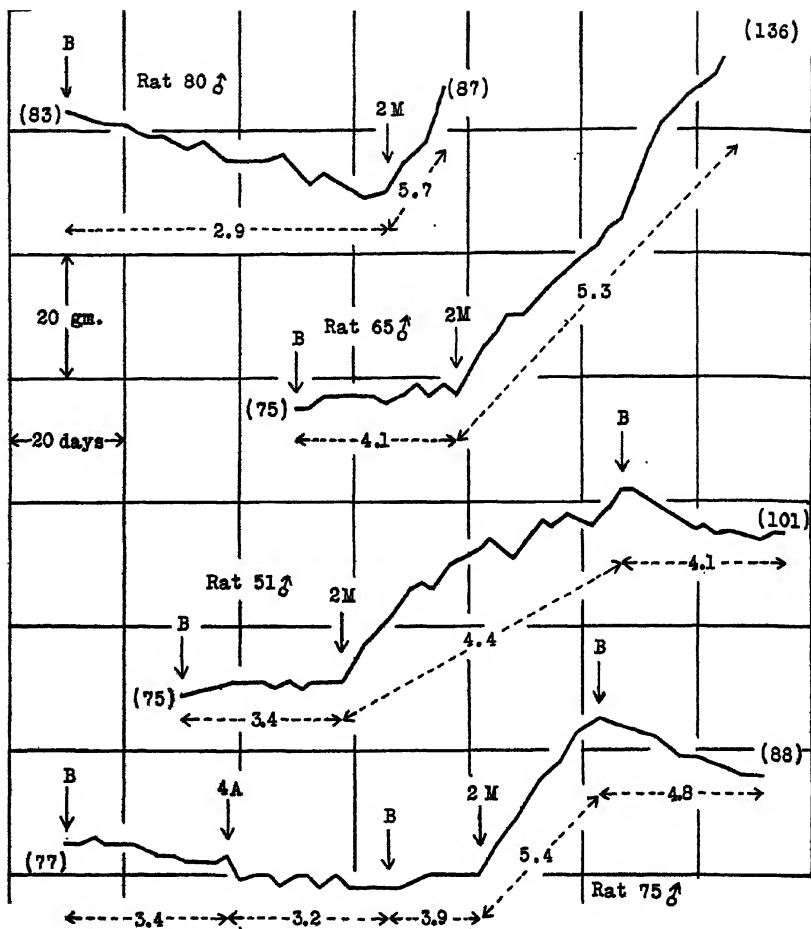


CHART I. Growth on methionine and alanine. Rats 80, 65, and 51: growth on the basal cystine-deficient Diet B (B) and on Diet B with 2 equivalents of methionine (2M); in the case of Rat 51, growth finally on Diet B (B). Rat 75: growth on Diet B (B); on Diet B with 4 equivalents of alanine (4A); on Diet B (B); on Diet B with 2 equivalents of methionine (2M) and finally on Diet B (B).

The diet employed during any part of an experiment is indicated on the graph by letter and number notation (see text) with a downward arrow at the point representing the beginning of the period. The average daily food consumption in gm. for the corresponding interval is shown by figures inserted between arrows constructed with broken lines. The slants of the broken lines possess no significance. The initial and final body weights in

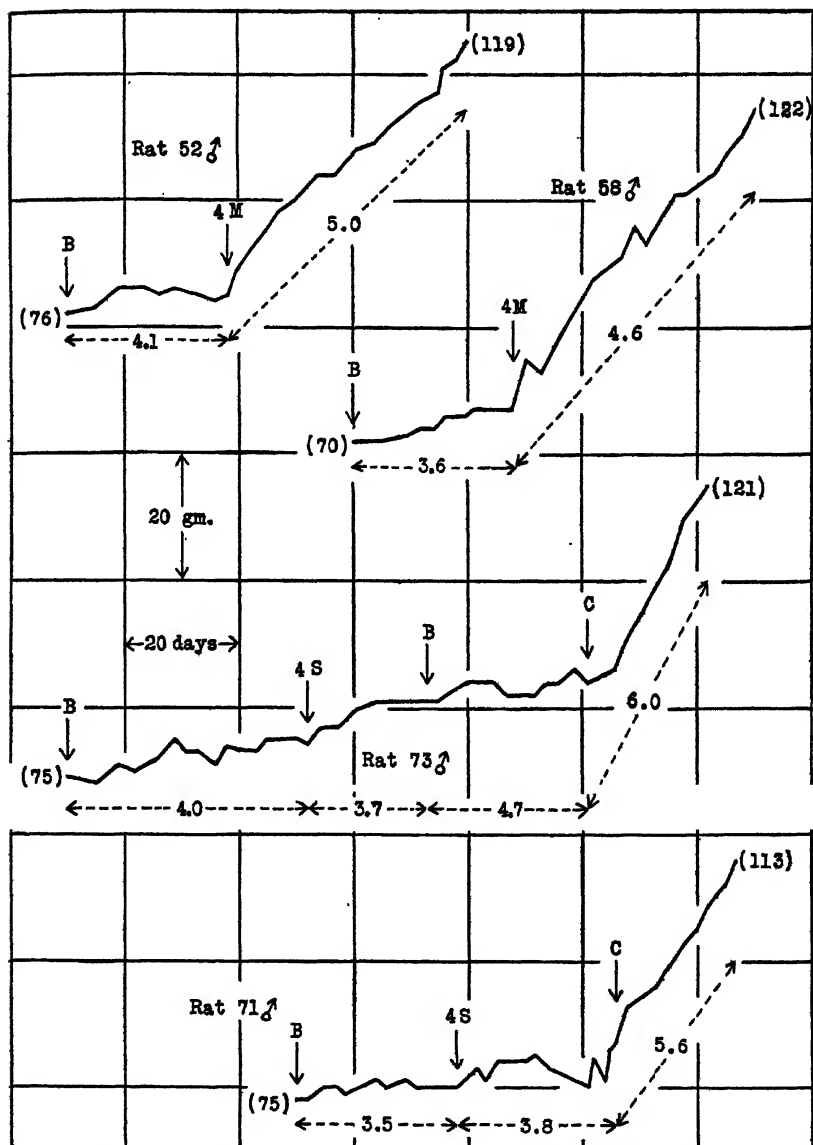


CHART II. Growth on methionine, serine, and cystine. Rats 52 and 58: growth on Diet B (B) and on Diet B with 4 equivalents of methionine (4M). Rat 73: growth on Diet B (B); Diet B with 4 equivalents of serine (4S); on Diet B; and finally on Diet B with 1 equivalent of cystine (C). Rat 71: dietary changes similar to those for Rat 73. The designations for diet employed, food consumption, and body weights are the same as in Chart I.



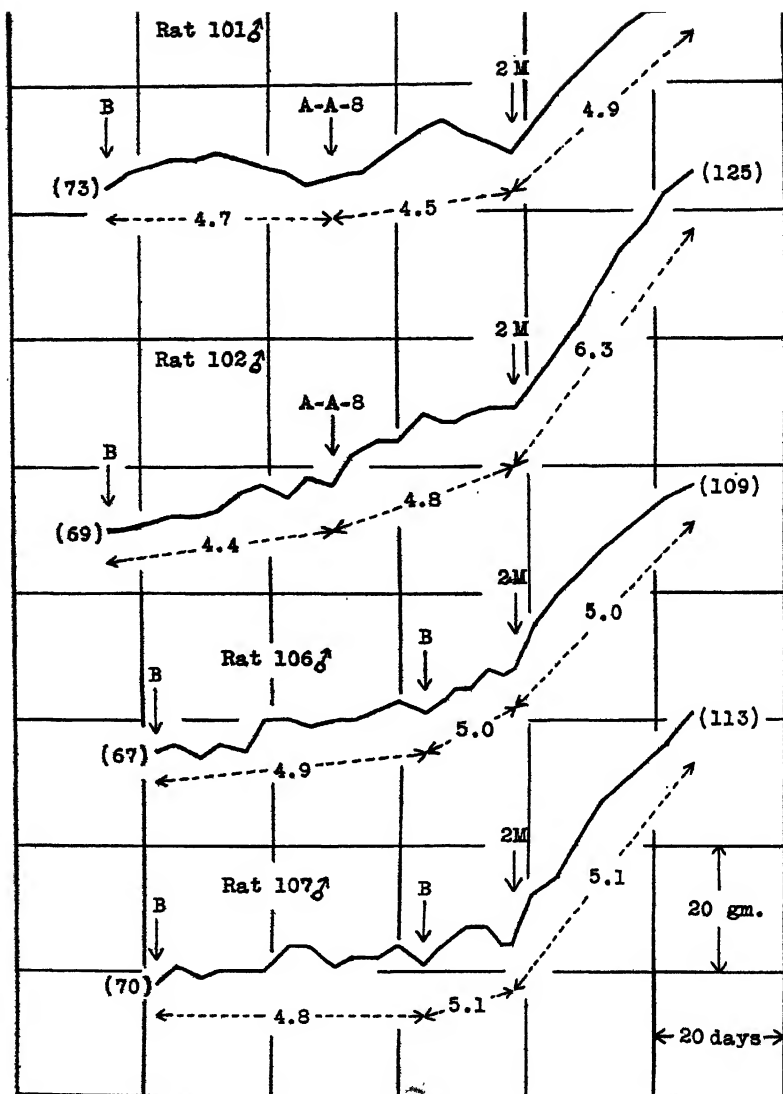


CHART III. Growth on a supplement of eight amino acids without cystine and methionine, on methionine, and on methionine with restricted food intake. Rats 101 and 102: growth on Diet B (B); on Diet B with a supplement of eight pure amino acids (A-A-8, see text) and finally on Diet B with 2 equivalents of methionine (2M). Rats 106 and 107: growth on Diet B (B) and on Diet B with 2 equivalents of methionine (2M), the food intake being restricted to those amounts which the two animals had respectively previously consumed of the basal diet (Diet B) alone.

other words, any one of the physiologically natural  $\alpha$ -amino acids might cause the same effect. Accordingly, tests were made with serine and alanine which are similar to cystine not only in being derivatives of propionic acid but also in giving rise to glucose in the diabetic organism. The growth curves of Rats 71, 73, and 75 demonstrate that these two substances produce no significant change in the animals' weights, although the subsequent administration of cystine or methionine caused immediate and continued body weight gain.

An additional control experiment of the same type was carried out with a supplement of eight different amino acids selected

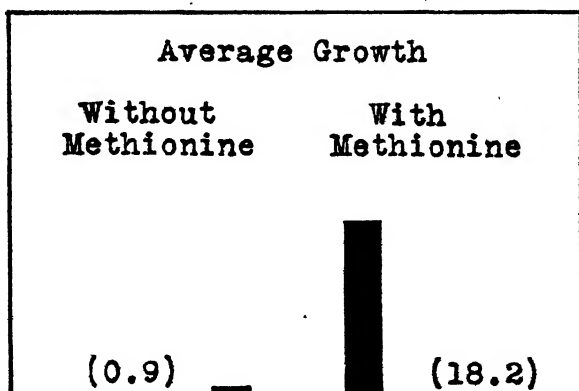


CHART IV. Comparison of the average growth in gm. of 10 rats (Rats 80, 65, 51, 75, 52, 58, 101, 102, 106, and 107) for 2 week periods immediately preceding and immediately following methionine administration.

from those which according to published analyses appear to constitute the lowest percentages of casein. They were each added in 0.5 molecular equivalents related to the standard of 120 mg. of cystine (1 equivalent) employed for supplementing 100 gm. of basal diet. The amino acids and the quantities used were as follows: glycine 38, alanine 45, hydroxyproline 66, phenylalanine 83, aspartic acid 67, serine 53, histidine monochloride 105, and tryptophane 102 mg. per 100 gm. of the basal diet. This combined supplement, like that of the alanine or of the serine, was without any continued appreciable effect on growth (see Rats 101 and 102, Chart III). The conclusion from these experiments is that cystine and meth-

ionine alone among a wide variety of amino acids cause a distinctly specific growth response when fed as a supplement to the basal diet under consideration.

*Controlled Food Consumption*—Certain aspects of the problem of the relation of food consumption to growth have been reviewed in a previous communication (Jackson, 1929; cf. Berg and Potgieter, 1931-32). Prolonged growth without adequate food consumption of the tryptophane-deficient diet then employed is apparently impossible. Hence, food was given *ad libitum* in the first of the present experiments in order that possible body weight gains of unquestionable magnitude might be registered. It will be observed from Charts I, II, and III that animals ingesting food *ad libitum* in every case consumed greater quantities of the diets supplemented with either methionine or cystine. Thus the relief of the deficiency under present discussion, like the amelioration of most, if not all, other nutritional deficiencies, results in an increased consumption of food by the large majority of animals.

To determine what effect the restriction of the intake of the methionine-supplemented diet might have upon growth, an experiment was performed with two animals, Rats 106 and 107 (Chart III). The food consumption was measured for 8 weeks in the ordinary way by means of spring scales calibrated in gm., and for the last 2 of the 8 weeks also by means of laboratory trip scales accurate within 0.2 gm. The latter measurement involving a total over-all possible error of not more than 5 per cent was used as a basis for restricting the food intake of the methionine diet for the next 4 weeks. During this time, the diet was weighed for a 4 day period and then divided into the four equal daily portions.

It is obvious that restriction of the food consumption in this instance does not prohibit methionine from exerting a marked growth-accelerating effect. The response is somewhat greater and more prolonged than in the aforementioned tryptophane experiments perhaps for the reason that the cystine-deficient animals were already growing at a somewhat greater rate than were the ones deficient in tryptophane. These findings constitute additional independent evidence in support of the general principle that growth stimulated in such instances with or without increased food consumption is not, as was vigorously contended to be possible by Mitchell (1927), the result of a direct *condimental*

influence upon appetite, but rather as Rose (1928) held, the indirect register of *increased chemical activity* in the cells brought about by enlarging the supply of an indispensable constituent heretofore available in amounts too small for optimum cell activity.

*Possible Interpretations*—Without further experimental evidence, we hesitate to assume that these results represent an actual conversion of methionine to cystine, though the possibility of such a transformation seems clearly indicated. It should be emphasized that the animals acquired a small but definite intake of both cystine and methionine as well as a certain amount of as yet unidentified sulfur from the proteins of the whole milk powder and presumably also from the yeast. With the latter fact in mind, one may picture the various possibilities as follows: (1) That both cystine and methionine are indispensable; and that with the type of diet used either cystine or methionine supplementation permits growth of the kind depicted in the accompanying charts. Such a circumstance, however, seems unlikely in view of data described in the literature—for example, Osborne and Mendel's finding as to the tryptophane-lysine requirements of animals ingesting zein, indicating that an animal deficient in two necessary substances cannot grow substantially without a supplement of both missing factors. (2) That cystine and methionine make up a freely interconvertible system of which only one member is necessary. (3) That both amino acids are indispensable but each only to a limited degree, and that there is at least one metabolic function<sup>5</sup> which can be cared for by either amino acid or by some common metabolite of the two. This metabolite might conceivably consist of a very small fragment, such as divalent sulfur of such an origin that it could be generated at the proper place and time. (4) That only one of the amino acids, say cystine, is indispensable to a limited degree and that an adequate supplement of the other, in this instance, methionine, may suffice for a common function similar to that indicated under item (3).

<sup>5</sup> The various major and subordinate functions which cystine may serve in the body have been dealt with by various writers (*cf.*, *e.g.*, Garrod, 1923; Lightbody and Lewis, 1929; and Abderhalden and Wertheimer, 1931). Apparently cystine is a precursor or one of the precursors of glutathione, taurine, certain detoxication products, etc., and of insulin, keratin, and other important proteins of the body.

The foregoing hypotheses are all premised on the condition that the metabolic relation of cystine and methionine is circumscribed *within* the body. Yet it has been clearly recognized by various writers that a substance administered orally is always subject to the action of alimentary bacteria. And indeed, if methionine can be synthesized into cystine *in vivo*, the lower forms of life with their almost unlimited capacity in general to bring about structural modifications of organic compounds might more readily be expected to achieve the methionine-cystine transformation. Rimington and Bekker (1932) have reviewed the literature bearing on the phenomenon of sheep being able to deposit cystine in their wool to the extent of several times the amount detectable in the pasturage ingested, and have raised the question as to the possible rôle of bacteria in the gut in converting inorganic sulfur to cystine. These authors, however, lay particular stress on the very favorable conditions for microbiotic activity in the intestinal tract of sheep as contrasted to those in the gut of omnivorous animals such as the rat.

Other speculative conceptions may be elaborated, but those outlined above will serve to show that the relation between the two amino acids may be complicated, and cannot be definitively ascertained from the experimental data submitted at this time. Further study is necessary to permit a precise interpretation of the facts at hand. The successful arrangement of a sulfur-free diet would make it possible to provide the answers to many of the questions to which attention has been directed. Another mode of attack is to study the excretion of bromophenylmercapturic acid following the administration of methionine. A definitely increased output of this product would constitute good evidence of a conversion of methionine to cystine. It will be interesting also to investigate the fate of methionine in the cystinuric individual. It is well known that the cystine output of cystinuric subjects is not increased by the administration of cystine itself but that it is augmented by the ingestion of protein. Is it possible that the cystine originating from protein in the cystinuric individual actually has its origin in the methionine present in the protein? A successful demonstration of such a relation would again be tantamount to proof that the body can convert methionine to cystine.

It is obvious, of course, that, since the addition of methionine to

the diet of animals subsisting on the regimen previously described leads to growth stimulation, the study of the physiological behavior under similar conditions of  $\gamma$ -methio- $\alpha$ -hydroxybutyric acid, other sulfur acids, and the separate optically active forms of methionine becomes important. We have already submitted a tentative report on the first of these problems (Block and Jackson, 1932).

#### SUMMARY

1. Methionine, like cystine, is capable of unmistakably stimulating growth in albino rats subsisting on a basal diet poor in cystine.
2. Other amino acids, including serine and alanine, are ineffective in this regard.
3. The restriction of the food intake to a constant amount throughout the experiment does not prevent growth acceleration as a result of methionine supplementation.
4. Various interpretations of the experimental findings have been discussed.

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## INORGANIC SALTS IN NUTRITION

### IV. CHANGES INDUCED IN THE BLOOD BY A RATION DEFICIENT IN INORGANIC CONSTITUENTS\*

By PEARL P. SWANSON† AND ARTHUR H. SMITH

(From the Laboratory of Physiological Chemistry, Yale University,  
New Haven)

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The study of the rôle of the inorganic salts in nutrition has been approached in only a comparatively few cases by experiments dealing with the adjustments made by the animal organism when the ash constituents are withdrawn as completely as possible from an otherwise adequate dietary mixture.

60 years ago, Forster (1873) demonstrated that a diet devoid of all inorganic matter is incompatible with life and health. Lunin (1881), however, suggested that the experimental results reported by Forster were due, not to a deficiency of the mineral elements in the diet, but to an acidosis produced by the catabolism of the proteins. Upon testing this hypothesis, Lunin found that mice lived for only a short time on a salt-poor ration which had been rendered basic in reaction by the addition of sodium carbonate. Taylor (1904) also believed that the diuresis, diaphoresis, muscular twitchings, and painful movements that developed when he lived upon a diet free from all ash constituents were due to the potential acidity of the diet. In 1908, Goodall and Joslin repeated this experiment and, for the most part, confirmed the effects on the human being of subsisting upon food devoid of the inorganic salts. Osborne and Mendel, in 1911, demonstrated the decline of rats upon a ration low in salts; however, they were unable to

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\* The data reported in this paper are taken from a dissertation presented by Pearl P. Swanson in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University, 1930.

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† Alexander Brown Coxe Fellow, Yale University, 1929-30.



induce a positive response when the missing constituents were added to the diet.

These early experiments were complicated by the fact that certain essentials other than the mineral elements were also missing from the experimental rations. Not until 1923 were studies reported in which a complete withdrawal of salts constituted the sole dietary deficiency in a ration otherwise adequate and constituted of isolated foodstuffs. At that time, Osborne and Mendel found that young rats ceased to grow when the total amount of an adequate salt mixture was limited to 0.5 or even to 1.0 per cent of the test diet. Their results again suggested the possibility of producing a more or less permanent defect by such a procedure, for recovery did not always occur when the salts were again incorporated into the diet.

In 1927, Winters, Smith, and Mendel made a more intensive study of the effect on the rat of limiting the total intake of a qualitatively satisfactory salt mixture. As in former experiments, the growth of the newly weaned rats employed in the study was definitely retarded. In spite of the stunting that occurred, there was a persistent skeletal growth which was not accompanied by a proportional increase in the weight of certain bones. In studying the development of other body parts, such as the kidney, testes, liver, and heart, in animals receiving the defective diet, these investigators found that "the most significant change brought about in the organs was a marked and consistent increase in the kidney weight" (p. 592). The dissociation of growth in length and weight, the extremely slight increase in weight of certain bony parts, and the hypertrophied kidney all suggested that the animal must be making unusual adjustments to meet the stringencies of this rigid nutritive régime.

This point of view gained credence when Smith and Schultz (1930) demonstrated that an unusual polycythemia characterized the blood of young rats maintained for 1 month upon the low salt diet formulated by Winters, Smith, and Mendel.

Smith and Schultz, however, did not attempt to define the nature of the induced polycythemia. For instance, the term, polycythemia rubra, signifies an increased number of erythrocytes per unit of circulating blood. The condition may represent either an increase in the number of cells per unit volume due to a decrease in total blood volume, or it may indicate an absolute increase in the number of cells in circulation. The first state is known as a *relative* polycythemia; the second is called an *absolute* or *true* polycythemia. The present study was designed primarily to determine whether the augmented number of erythrocytes per unit volume found in the blood of the rats maintained on a low salt ration represented a relative or a true polycythemia.

Again, Smith and Schultz presented certain data indicating that

the red blood cells, although present in great numbers, were strikingly subnormal in size. These findings were particularly significant, inasmuch as considerable evidence has accumulated recently to the effect that the size of the red blood cell is an index of the adjustment of an organism to a state of anemia (Haden, 1925; Price-Jones, 1922; Mayerson and Laurens, 1931; and Leichsenring and Hönig, 1931). Further verification of the size of the red cell in the blood of the rat maintained upon a ration poor in salts seemed advisable, for the experimental conditions herein described were such as might induce osmotic effects capable of affecting the dimensions of the cell.

Furthermore, it is known that although the erythrocytes in pernicious anemia are not supersaturated with hemoglobin, the individual cells frequently contain more pigment than do normal cells (Wintrobe, 1929). On the other hand, due to the absolute decrease in the number of red blood cells characteristic of this disease, the total amount of circulating hemoglobin is diminished. The augmentation in the number of erythrocytes in the blood of the animals given the low salt ration is undoubtedly associated with changes in the total quantity of circulating pigment. However, the degree or the mechanism of this response at the time of the initiation of this study was not clear.

The studies implied above required more blood than was available in the earlier experiments, which made it necessary to study the effect of the withdrawal of dietary salts on a large size animal. The chief objectives of this study were, therefore: (1) to render a nearly mature rat polycythemic by the administration of a ration deficient in its mineral content only; (2) to determine by means of adequate criteria whether the induced polycythemia is relative or absolute; and (3) to establish the relation between the number of the erythrocytes, their size, and the hemoglobin concentration of the blood.

### *Plan of Experiment*

Several litters of vigorously growing young rats, reared for a time after weaning upon a stock ration rich in minerals and vitamins, were divided into three groups. In so far as possible, representatives of each litter were placed in each group. During the experimental period of 90 days, Group I received a synthetic

ration (Diet 3) which, according to present day standards, was deficient only in its content of mineral constituents. These rats maintained a uniform body weight for the last 60 days of the interval. The animals of Group II were fed a diet identical to that consumed by Group I except that it contained a satisfactory salt mixture (Osborne and Mendel, 1917). These rats were likewise continued upon the ration for 90 days and were, therefore, designated as the *chronological* or *age* controls. At the end of this period, individual chemical and morphological studies were made on the bloods of the members of each group of animals.

The third group of rats served as the *physiological* or *weight* controls. They were grown upon the same adequate ration fed to Group II until they weighed 160 gm., the body weight characteristic of the rats on the low salt ration. The blood of these rats was also examined. Thus, with two sets of controls, it was possible to compare the blood of the rat reared upon the ash-poor diet not only with that of a normal rat of its own age but with that of a rat of similar body weight as well.

The number of erythrocytes, the volume occupied by them, and the respective concentrations of hemoglobin, plasma protein nitrogen, and total solids were determined. In addition, the total volume of circulating blood in the rats of each group was ascertained. Certain of these determinations, *i.e.* the number of erythrocytes per c.mm. of blood, the concentration of plasma protein nitrogen per 100 cc. of plasma, the per cent of total solids in the blood, and the total blood volume, serve as indices of the nature of the polycythemic condition in the rats grown on the low salt diets; the red blood cell volume determinations with calculations of corpuscular volume show the comparative sizes of the red cells in the blood of the three groups of rats; and the relative difference in the concentration of hemoglobin in the blood of the three groups indicates the correlation between lack of salts and pigment production. The number of observations comprising each individual study was sufficiently large to make statistical analyses possible.

#### EXPERIMENTAL

##### *Composition and Preparation of Diets*

Due to the fact that it was necessary in this study to deal with a rapidly growing animal with rich body reserves of all the essential

mineral elements, it was imperative that the inorganic salt content of the experimental diet be reduced to a minimum. This was accomplished by the purification of the casein, and by the proper choice of sources of the vitamin B complex. The two synthetic diets differed from each other only in the content of inorganic constituents.

Diet 3, the basal low salt ration, consisted of low ash casein, 18 per cent; dextrin, 55 per cent; Crisco, 27 per cent. The mineral constituents of the crude casein were removed by repeated washing with acidulated water (pH, 4.7). Diet 2, the adequate mixture, had the same composition as Diet 3, except that a salt mixture (Osborne and Mendel, 1917) replaced 4 per cent of the dextrin in the ration.

The original casein contained 3.86 per cent of ash. Analyzed by the official method (Association of Official Agricultural Chemists, 1919) at 450–500°, the purified protein was found to contain only 0.4 per cent of ash. When the casein was ashed with the use of calcium acetate in order to prevent the loss of volatile acid anhydrides by the method described by Shaw (1920), the per cent of ash residue was 1.87.

The basal diets were supplemented by the separate feeding of the following vitamin adjuvants: dried yeast, an alcoholic extract of wheat germ, cod liver oil, and wheat germ oil. It was difficult to choose an adequate source of vitamin B complex because materials rich in the adjunct were correspondingly rich in ash. The amount of yeast given daily, therefore, was reduced to 0.2 gm., fed in the form of tablets of uniform weight ( $0.2045 \pm 0.007$  gm.). This quantity was found to be sufficient for the promotion of adequate growth when supplemented by 1 cc. of an alcoholic extract of wheat germ (equivalent to 2 gm. of wheat germ).

The cod liver oil, a standardized product, was given at a level of 100 to 120 mg. (5 drops) per day and the wheat germ oil was given in 40 to 50 mg. quantities (3 drops).

The basal low salt diet, analyzed by the calcium acetate method, contained 0.51 per cent of ash. The daily level of the food consumption of the rats placed upon the defective diet was found to be approximately 7 gm. On this basis, the rat consumed in its basal ration 36 mg. of ash daily. In addition, the vitamin supplements introduced 30 mg. Thus, the total quantity of ash in the

food eaten each day by the rats on this diet approximated 66 mg. or 0.94 per cent of the diet.

### *Care of Experimental Animals*

Selected rats were grown for 9 days after weaning on a satisfactory ration composed of natural food materials. At the end of this period they weighed approximately 120 gm. They were then given the experimental diets and were housed in separate galvanized wire cages with raised bottoms of the type termed "new style" by Smith, Cowgill, and Croll (1925). In consideration of the fact that an important channel for the excretion of salts is through the intestine, it was particularly important to use this type of cage in order to prevent coprophagy. The cages were cleaned and cared for in the manner described by the above authors. In addition, special care was taken to remove all food which had collected in the seams and crevices. When a cage was first used, any food residues were removed with a probe. Thereafter, following sterilization, the inside of the cage was carefully scoured with a stout steel brush. This procedure has been found to be absolutely essential.

Rations were offered *ad libitum* in glass containers and a record of the food intake was kept. All vitamin adjuvants were fed apart from the basal rations in small porcelain cups containing a few drops of water. They were given six times per week with a double quantity on Saturday. Fresh supplies of distilled water were always present.

### *Methods*

*Drawing of Blood*—Blood was obtained by two methods. That needed for the erythrocyte enumeration and for the red blood cell volume and hemoglobin determinations was taken from the tail. In order to secure a peripheral dilatation of the vessels, the tail was first wiped with a piece of cotton saturated with toluene and then held in warm water for a few minutes. The tail was clipped, the first drop of blood discarded, after which samples for the count, hemoglobin, and red cell volume studies were taken. No determinations were made unless the blood was obtained from a freely flowing drop.

Large quantities of blood were needed for the estimation of

plasma protein nitrogen and of total solids in the blood. The method described by Swanson and Smith (1932) was used whereby it was possible to secure from the abdominal aorta of a normal rat as many as 6 cc. of blood that was entirely uncontaminated by body fluids.

*Enumeration of Erythrocytes*—A diluting chamber and a Neubauer counting cell, each certified by the Bureau of Standards, were used. The blood from the tail was considered a fair sample of the circulating fluid, for constant and consistent results were obtained in a given group of rats. Furthermore, the differences observed between groups were so great that the criticisms offered by Whipple and Robscheit-Robbins (1925) concerning this method for obtaining the sample do not apply here. Since all determinations were made in the same manner, the results are strictly comparable.

*Determination of Hemoglobin*—The acid-hematin method for the determination of hemoglobin (Cohen and Smith, 1919) was adopted in these studies. The Sahli pipettes used for obtaining the sample were calibrated with mercury. The sample in the acid solution was allowed to stand for 40 minutes at room temperature and was compared colorimetrically against a Newcomer disc under water, set at 6.5 mm. The Newcomer disc was standardized against the hemoglobin of dog blood by the method of Van Slyke (1918) for oxygen capacity.

*Determination of Red Blood Cell Volume*—The volume occupied by the red cells of the blood was determined by the method described by Van Allen (1925). It was found that changes in cell volume due to osmotic effects were eliminated by the use of a 1.6 per cent solution of potassium oxalate reported elsewhere (Cartland and Koch, 1928) as isotonic with the blood of normal rats. The 1.6 per cent oxalate solution was found also to be isotonic with the blood of the animals reared on the diet deficient in salts.

The manner of centrifuging was standardized for load and time required to bring the red blood cells to constant volume ( $\frac{1}{2}$  hour at 3000 R.P.M., 4 centrifuge cups). The same centrifuge was always used. Determinations were made in duplicate or triplicate and reported readings checked to 1 per cent.

*Determination of Plasma Protein Nitrogen*—The plasma nitrogen was determined by the procedure outlined by Swanson and Smith (1932). Duplicate or triplicate analyses were made on the plasma obtained from each rat.

*Determination of Total Solids*—As soon as the blood was withdrawn from the artery of the rat, it was placed in a tared glass weighing bottle and covered. It was weighed immediately to 0.1 mg. and then dried at 105° to constant weight in an electric oven.

*Determination of Blood Volume*—The method used for the determination of the absolute blood volumes of the rats reared upon the experimental rations was that of Went and Drinker (1929). It was modified in several ways, however, for it was necessary to use every precaution to obtain true values for red blood cell volume and for the perfect matching of the samples of blood containing the dye, brilliant vital red, with the standard color tubes. In order to make satisfactory color comparisons, the capillary tubes used in preparing the standard color scale, and for receiving the sample of blood, must be of exactly the same bore. They were tested from both ends by the capillary rise of ether against one tube of the desired diameter. The use of the preliminary sample of blood recommended by Went and Drinker was abandoned in order to eliminate blood volume changes that may result from tail bleeding. Difficulty was also experienced in obtaining accurate values for red blood cell volume with the capillary tubes used by Went and Drinker. The Van Allen hematocrit method already described was, therefore, substituted. This was possible when the final sample of blood was secured from the abdominal aorta. After the injection of the dye into the femoral vein, a mixing time of 3½ minutes was allowed; then the artery was exposed with all precautions to avoid bleeding. Exactly 4½ minutes after the injection of the dye, blood was withdrawn by means of a syringe. One sample in the capillary tube, two samples for red blood cell volume in the Van Allen tubes, and a final check sample in a capillary tube were taken from the syringe in this order. It was necessary to use the lightest colored heparin obtainable (a gray-white powder) for the commonly used darker product can so alter the color of the plasma that perfect color comparisons are impossible. The color of the plasma-dye sample was always matched against the standard color scales by two persons (A. H. S. and P. P. S.) for a variation in the reading of 0.1 per cent introduced erroneous values.

*Results**Growth*

When the inorganic constituents were removed from the ration of a rat weighing approximately 120 gm. at the beginning of the experimental period, the animal grew at a subnormal rate for some 32 days. Thereupon, the growth response was inhibited to such an extent that no further increments in growth occurred and the body weight was maintained at approximately 160 gm. for the remainder of the experimental period. Such maintenance of a constant body weight for a period of at least 8 weeks is absolutely essential if a characteristic blood picture is to be induced by subjecting the animal to the defective low salt diet. The length of time that elapsed before growth ceased and the level at which constant body weight was maintained were influenced by the early history of the animal. However, if the conditions of the pre-experimental period were standardized, the animal responded in a very uniform manner. The details of this standardization will be discussed in a later paper.

*Polycythemia*

One of the most striking changes that occurred on the low salt diet was the great increase in the erythrocyte concentration per unit volume of blood. The data are tabulated in Table I.

The average number of erythrocytes in 1 c.mm. of the blood of the rats reared on the salt-poor diet was  $12.0 \pm 0.12$  million in contrast to  $9.7 \pm 0.11$  million found in the blood of normal rats of the same age. With a probable error of this magnitude, the chances are twenty-one to one that other means drawn by random sampling from similar groups of animals will lie in the range, 11.8 to 12.2 million. Similarly, the mean number of erythrocytes in the blood of normal rats may be expected to fall in the range, 9.59 to 9.81 million. The difference in the mean number of red blood cells, i.e. 2.3 million, in the bloods of the two groups of animals may be ascribed with absolute certainty to the diet  $\left( \frac{d.}{p.e.d.}, 14.4 \right)^1$ . Normal animals of the same body weight, but

<sup>1</sup> In the ratio,  $\frac{d.}{p.e.d.}$ , d. is the difference between means and p.e.d. is the probable error of the difference.



TABLE.  
*Comparison of Certain Constituents of Blood of Rats Reared upon Certain Experimental Diets*

Blood study	No. of animals in control and low salt groups			Means with their probable errors*					Significance ratios†		
				Physiological		Chronological		Low salt			
	Chronological	Food <i>ad libitum</i>		Food restricted	Food <i>ad libitum</i>	Food restricted	Low salt and physiological controls		Low salt and chronological controls		
		Food <i>ad libitum</i>	Food restricted					Food <i>ad libitum</i>	Food restricted		
Erythrocytes, millions per c.mm. ....	28	25	23	26	7.7 ± 0.10	9.7 ± 0.08	9.2 ± 0.08	12.0 ± 0.12	27.2	14.4	19.2
Red blood cell volume, per cent. ....	22	20	23	22	48.0 ± 0.35	54.0 ± 0.46	53.0 ± 0.28	45.0 ± 0.30	6.6	16.4	19.6
Hemoglobin, gm. per 100 cc. ....	25	25	24	27	15.4 ± 0.24	17.4 ± 0.19	16.5 ± 0.28	14.3 ± 0.22	3.4	10.7	6.2
Total solids, per cent. ....	19	25	19	25	19.3 ± 0.20	20.6 ± 0.23	21.1 ± 0.19	19.0 ± 0.31	1.0	4.4	5.8
Plasma protein N, gm. per 100 cc. ....	21	21	16	27	1.01 ± 0.01	1.14 ± 0.01	1.11 ± 0.01	1.06 ± 0.01	3.0	4.5	2.9

\* See Fisher (1930) for treatment of small samples.

† The significance ratio is expressed by  $\frac{d}{p.e.d.}$  where d. is the difference between means and p.e.d. is the probable error of the difference. The difference between two means is considered statistically significant when  $\frac{d}{p.e.d.}$  is 3 or greater.

younger than the stunted animals, *i.e.* the physiological controls, had  $7.7 \pm 0.10$  million red cells per c.mm. of blood. Compared with the average of  $12.0 \pm 0.12$  million, characteristic of the blood of the rats reared on the ration poor in salts, the difference is highly significant  $\left( \frac{d.}{p.e.d.}, 27.2 \right)$ .

As is true of animals consuming any defective diet, the rats on the low salt regimen consumed a smaller quantity of food per day than did the rats on the adequate ration. The food consumption in gm. of the twenty-six rats on the ash-poor diet averaged daily during the successive weeks of the experiment, 8.1, 7.0, 7.0, 6.6, 6.2, 5.8, 5.8, 5.9, 6.0, and 5.5, whereas the rats on the adequate diet consistently consumed daily an average of 10 gm. of food throughout the entire experimental period. Therefore, in order to limit the variables and to prove that the polycythemia observed in the rats fed the low salt ration was not related to a reduced caloric intake, the bloods of the members of another series that was essentially a paired feeding control group were studied.

Twenty-three animals received the adequate ration (Diet 2), fed week by week, in the quantities which we had already found to be the average weekly intake of food of the rats on the deficient diet. Upon this restricted diet, the final weight of the animals was about three-fourths of that attained by the rats that consumed the same food *ad libitum*. An enumeration of the red cells in the blood of these rats, showed that the average number per c.mm. was 9.2 million. This figure is significantly different  $\left( \frac{d.}{p.e.d.}, 3.7 \right)$

from that representing the concentration of erythrocytes in the blood of the rats consuming the adequate food in unrestricted quantities. Indeed, the difference represents a trend toward an anemic rather than toward a polycythemic condition. This fact is further emphasized by the values of the significance ratios, *i.e.* 14.4 and 19.2, respectively, obtained when the mean red blood cell count of the rats on the low salt diet is compared with those of the two chronological control groups receiving food *ad libitum* and in restricted quantities.

The low plane of food consumption of the rats on the salt-poor ration automatically reduced their intake of protein. It seemed possible that this factor might be associated with the production

of the "low salt polycythemia." Therefore, another diet<sup>2</sup> was formulated in which the percentage of protein in the ration low in salts was increased so that the animals would receive even on their reduced caloric intake the equivalent of the quantity of protein consumed daily by the rats eating the adequate diet *ad libitum*. The blood of the rats fed this diet was found to contain on the average the same number of erythrocytes (11.8 million) as was present in the blood of the rats receiving Diet 3. Therefore, the decreased ingestion of protein occurring concomitantly with the consumption of the low salt diet was not responsible for the increased number of cells in the blood of the rats maintained on the defective ration.

On the basis of these experiments in which the variables were ruled out that were introduced by the characteristic decrease in the daily intake of food of the rats placed on the low salt diet, one must conclude that the polycythemia of the rats fed the low salt ration represented an adjustment of the animal body to the prolonged absence of the inorganic components of the diet.

Values for red blood cell counts above the average have been induced by experimental means previous to the preliminary report of this investigation (Smith and Swanson, 1929). For instance, the erythropoietic actions of phosphorus, germanium, arsenic, cobalt, and zinc have been observed when these elements are added to the food of experimental animals (Hammett, Nowrey, and Müller, 1922; Harrop, 1928; Waltner, 1929; Drinker, Thompson, and Marsh, 1927). The stimulating effect of certain of these metals in the remission of nutritional anemia, *i.e.* cobalt, zinc, germanium, when fed with iron, has been confirmed recently by Myers, Beard, and Barnes (1931-32) who, in addition, noted exaggerated erythropoiesis when vanadium or manganese alone supplemented a stock diet. Orten, Underhill, Mugar, and Lewis (1932), on the other hand, could only induce a polycythemia with cobalt when copper was added as well. Exposure to various chemical agents and pharmacologically active substances is known also to produce polycythemia in industrial workers. The more common of such substances are arsenic, phosphorus, manganese, mercury, radium, and iron (Harrop, 1928).

<sup>2</sup> High protein-low salt diet, Diet 4: low ash casein, 27 per cent; dextrin, 46 per cent; Crisco, 27 per cent.

A chronic state of anemia maintained by hemorrhage is characterized by a red blood cell count of double the original value after 11 weeks, according to Mayerson and Laurens (1931).

On the other hand, descriptions of an increased number of erythrocytes in the blood, directly traceable to a dietary deficiency, are very rare. Only two such instances have been found. Scott, in 1923, found an increase in erythrocytes in the blood of young rats reared by mothers that had been fed a diet of bread and milk for 10 months. However, recent studies in nutritional anemia do not indicate similar findings (Hart *et al.*, 1925; Mitchell and Schmidt, 1926; Beard and Myers, 1931-32). The second instance of a high erythrocyte count as the result of a dietary adjustment is the isolated case cited by Happ (1922), wherein the red corpuscle count of a rat reared by a mother on a diet low in iron was 12.2 million in contrast to a normal count of 7.7 million.

#### *Is the Polycythemia Relative or Absolute?*

Judged by indices such as the concentrations of the total solids and of plasma protein nitrogen, the blood of the polycythemic animals was definitely more dilute than was that of the normal animals (Table I). The conditions of the experiment have interfered with the normal processes of dehydration, for the concentrations of both of these constituents were significantly less in the blood of the rats deprived of their dietary mineral elements than they were in the normal rats of the same age. Moreover, a high degree of certainty can be attached to the validity of each difference  $\left( \frac{d.}{p.e.d.}, \text{total solids } 4.4; \frac{d.}{p.e.d.}, \text{plasma protein nitrogen } 4.5 \right)$ .

Thus, the data strongly suggest that the polycythemia induced by a low salt dietary régime is one associated with a hydremia, rather than with the anhydremic condition described by Parkes Weber and Bode (1929) as an essential corollary of a true polycythemia.

Although the "hydremic polycythemia" induced in the rat by our defective diet is rather a paradoxical condition, it is entirely conceivable that the hydremic state may be due to a disturbance of the osmotic forces operating when an animal exists for a long period of time largely on endogenous sources of necessary mineral matter. In most instances, the concentration of nitrogen in the plasma is a very useful index of the concentration of blood, par-

ticularly when used in conjunction with some other determination. However, there are times when low values for plasma protein nitrogen do not indicate hydremia. The most important of these from the present standpoint is the dilution of the plasma that results from diets very low in protein (Frisch, Mendel, and Peters, 1929). It is to be emphasized at this point that the low value obtained for protein nitrogen in the plasma of the stunted rats was not due to a protein starvation. This is proved by the fact that the plasma protein concentration was not significantly reduced from normal

TABLE II  
*Blood Volumes of Normal Rats and Rats on Low Salt Ration*

No. of observations	Physiological controls		Chronological controls		Low salt rats	
	Total	Per 100 gm. body weight	Total	Per 100 gm. body weight	Total	Per 100 gm. body weight
	cc.	cc.	cc.	cc.	cc.	cc.
1	12.2	7.6	20.0	7.2	11.1	6.4
2	12.6	7.7	21.0	7.0	13.8	7.9
3	12.4	7.8	20.5	7.4	12.2	6.4
4	12.6	7.8	19.0	6.9	11.4	6.0
5	12.4	7.6	22.6	7.6	12.2	6.7
6	12.9	7.8	18.5	7.1	11.9	7.0
7	11.9	7.6	18.0	7.1	12.0	8.1
8	12.1	7.7			11.4	7.6
9	12.3	7.6			11.6	7.8
10	10.6	6.7			12.3	7.4
11	12.9	8.1				
Average...	12.3	7.6	19.9	7.2	12.1	7.1

$\left( \frac{d.}{p.e.d.}, 2.0 \right)$  in the "restricted chronological" control group, which had consumed exactly the same quantity of protein as had the rats on the low salt diet.

Furthermore, the polycythemia herein described is not the result of any decrement in the total volume of circulating blood of the rats receiving the salt-poor food (Table II).

Data on two groups of normal rats are reported: those on young rats approximately 49 days old and on older rats, 130 days old.

These groups represented the physiological and chronological groups in respect to the series that was subjected to the ration low in salts. As in normal human subjects (Rowntree, Brown, and Roth, 1929), the relative volume of blood in normal rats is a fairly constant figure. The individual volumes of blood found in the physiological controls were surprisingly consistent. The average total blood volume of these rats was 12.3 cc. and the average volume per 100 gm. of body weight was 7.6 cc. The uniformity of the results may be explained by the fact that these rats were selected arbitrarily at a body weight of 160 gm. and deviations were eliminated that normally occur in a group of individuals due to differences in body structure (Rowntree *et al.*, 1929).

The total amount of circulating fluid in the body of the chronological control was 19.9 cc. The average weight of the animals was 280 gm. Expressed in terms of body weight, the volume of blood per 100 gm. of body weight (7.2) was only a little less than that (7.6) found in the physiological control animal. This difference probably is not significant. If it is, two physiological factors may be responsible; *i.e.*, the relative ages of the two series of animals and the relative sizes of their depots of body fat (Darrow, Soule, and Buckman, 1928). The average value of the blood volumes of all normal rats herein recorded is exactly that, 7.4 per cent of the body weight, reported by Went and Drinker in 1929. This figure is considerably higher than the value given by earlier workers as the relative blood volume of this animal.

It is worthy of note that the relative blood volume of the rat is not affected by a dietary régime in which the restriction of salts represents the limiting factor. The rats on the low salt diet had 7.1 cc. of blood per 100 gm. of body weight; the physiological controls, 7.6; the chronological controls 7.2. In the two groups where the average body weight of each group is the same, *i.e.*, the rats fed the low salt ration and the physiological controls, the total blood volumes are also practically identical (12.1 and 12.3 cc.). The polycythemia observed can, therefore, be regarded as a true polycythemia.

#### *Size of Erythrocytes*

Although the number of erythrocytes increased greatly during the period in which the experimental rats were maintained on the

low salt ration, the data presented in Table I indicate that at the end of an experimental period of 90 days the size of the red blood cells had diminished considerably, for the relative volume occupied by them is less than that of the red blood cells in any of the control series. For instance, the average volume of the erythrocytes in the blood of the various groups was as follows: with 12 million cells per c.mm. of blood in the rat on the low salt diet, 45 per cent; with 9.7 million cells in the chronological control, 54 per cent; with 9.2 million cells in the restricted chronological control, 53 per cent; and with 7.7 million cells in the physiological control, 48 per cent. When the volume of erythrocytes in the blood of the rats given the ration poor in salts is compared with each control series, the ratios  $\left(\frac{d.}{p.e.d.}, 6.6., 16.4., \text{ and } 19.6\right)$  express absolute certainty that the differences between the groups are significant. Even the smallest ratio, 6.6, obtained when the average red blood cell volume of the rats on the low salt diet is compared with that of the physiological controls is highly significant.

With the data available, it is possible to calculate the average volume occupied by the red blood cells under these various experimental conditions. Wintrobe (1929) has suggested that the size of the cell can be better indicated by *corpuscular volume* than by *volume index*. Corpuscular volume in terms of cubic microns, or  $10^{-12}$  cc. is obtained by the determination of the value of the ratio,

volume (cc. per 100 cc.)

red blood cell count (in millions). Thus, the average corpuscular volume of the cells found in the blood of the rats fed the low salt ration is 37.5 cubic microns in contrast to a cell volume of 56 cubic microns in the chronological controls, 58 cubic microns in the restricted chronological controls, and 62 cubic microns in the physiological control. Under normal conditions then, the red blood cell in the young rat (49 days) is somewhat larger than it is in the older rat (127 days). The data show further that the corpuscle of the normal rat of the same age as the rat fed the ration low in salt is 1.5 times larger than the cell of the stunted rat and the corpuscle of the physiological control, 1.7 times larger.

The earlier observation of Smith and Schultz (1930) on the decreased size of the red blood cell in the blood of 40 gm. rats, as determined by actual measurement of cell diameter, is in accord

with the volume as herein calculated. The small cell is undoubtedly a reflection of the absence of blood-forming elements in the diet. In hemorrhagic anemia in which the hemoglobin concentration is maintained at a constant but subnormal level by repeated bleedings, the red blood cells become diminutive when the experimental diets employed are not satisfactory for blood regeneration (Mayerson and Laurens, 1931; Leichsenring and Hönig, 1931). Foster (1931) has recently found that a small cell characterizes nutritional anemia as well.

### *Hemoglobin*

The concentration of the pigment in the blood of the rats given the low salt ration showed a definite decrease (Table I). The anemia was not as pronounced as that which develops when rats are fed on a diet of milk alone. However, in relation to the chronological controls, the animals are distinctly anemic ( $17.4 \pm 0.19$  gm. per cent *versus*  $14.3 \pm 0.22$  gm. per cent). The concentration of hemoglobin in the blood of the rat on the low salt ration is also significantly lower ( $\frac{d.}{p.e.d.}, 3.4$ ) than that in the blood of the physiological controls. On the absolute basis, the blood of the anemic animal contains 1.7 gm. of hemoglobin in contrast to 3.5 gm. present in the blood of normal animals of the same age and 1.9 gm. for normal animals of the same weight.

Again, the calculation of corpuscular hemoglobin (Wintrobe, 1929) will picture clearly the quantity of the hemoglobin present in each cell. This value is calculated from the ratio, Hb (gm. per 1000 cc.) to red blood cell count (in millions).

The absolute quantity of hemoglobin in the erythrocyte of the rat fed the low salt diet is found to be definitely low. In the three control groups, *i.e.* the "chronological *ad libitum*," the "chronological restricted," and the physiological, the absolute quantities of hemoglobin in the red blood cells were found to be  $18 \times 10^{-12}$  gm.,  $18 \times 10^{-12}$  gm., and  $19 \times 10^{-12}$  gm. respectively. However, even under the existing conditions of paucity of inorganic salts, the per cent of hemoglobin in the cells of the experimental groups of animals was identical to that of the three control groups. This value is calculated by dividing corpuscular hemoglobin by corpuscular volume (Wintrobe, 1929). The per cent saturation of



the cells of three control groups with hemoglobin was 32, 31, and 31.6; that of the cells of the low salt series, 31.6.

Reduction of total hemoglobin has been demonstrated before as the result of a nutritive deficiency. The most common instance is that of nutritional anemia. Of the many experiments in this field, the only results analogous to those of the present study are those of Scott (1923), who described an increased number of red blood cells with a lowered color index when animals were kept on bread and milk diets for two generations. Mayerson and Laurens (1931) working with dogs rendered anemic by hemorrhage according to the procedure of Whipple and Robscheit-Robbins, also describe a similar decrease in the quantity of corpuscular hemoglobin after an experimental period of 11 weeks. They call attention to a similar situation in certain of the experiments of Whipple and his collaborators (Elden, Sperry, Robscheit-Robbins, and Whipple, 1928). It is of interest to note that Mayerson and Laurens found in the dog, as we did in the rat, that the percentage saturation of the cells with hemoglobin is the same in anemic and normal animals.

#### SUMMARY

By the elimination of practically all of the inorganic constituents of the diet, an atypical blood picture was produced in a rat maintained at a constant body weight by the dietary adjustment.

The number of erythrocytes was greater than normal in reference to both animals of the same age and those of similar body weight.

That the condition was a true polycythemia, was indicated by a normal blood volume and a slight hydremia; no concentration of the circulating fluid had occurred.

The cells were very small in size as shown by determinations for red blood cell volume and by calculations for corpuscular volume.

The total hemoglobin was reduced as was also the absolute quantity in the individual cell. However, the percentage of hemoglobin in each cell was normal.

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## INORGANIC SALTS IN NUTRITION

### V. PROGRESSIVE CHANGES IN THE BLOOD OF RATS MAINTAINED UPON A RATION POOR IN INORGANIC SALTS\*

By PEARL P. SWANSON† AND ARTHUR H. SMITH

(From the Laboratory of Physiological Chemistry, Yale University, New Haven)

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It has been shown (Swanson and Smith, 1932, *b*) that an absolute polycythemia may be induced in rats by maintaining them for 90 days on a ration in which the total ash of the diet and of its supplements has been reduced to 0.94 per cent. The blood is slightly hydremic and the volume of the circulating fluid is normal for a rat of the size of the experimental animal. The red blood cells at the end of the experimental period are very small and the quantity of hemoglobin is less than normal. Inasmuch as data on the development of the syndrome during the experimental period would furnish additional information concerning the physiological adjustment of the animal to the unfavorable dietary régime, the study herein described was carried out.

When the experimental animals weighed 120 gm., they were placed on the low salt and on the adequate rations described in Paper IV (1932, *b*) of this series. The necessary precautions had been observed in the selection of the animals and in their care during the preexperimental period. The erythrocytes were enumerated in the blood of the rats comprising each group at 3 week intervals. In addition, the volume occupied by the red blood cells, the hemoglobin concentration, the per cent of total solids, and the con-

\* The data reported in this paper are taken from a dissertation presented by Pearl P. Swanson in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1930.

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† Alexander Brown Coxe Fellow, Yale University, 1929-30.

centration of nitrogen in the plasma were determined at the same time.

Ten rats were studied at the beginning of the experimental period. The average of these findings represented the initial picture of the blood for each group of rats. Changes occurring in the blood stream with advancing age were traced from this point in all rats. The bloods of nine normal rats on the adequate synthetic diet were examined after 21 days, eight after a period of 42 days, and eleven after 63 days. Likewise, thirteen, thirteen, and ten rats, respectively, fed the low salt ration, were studied at the same intervals of the experimental period. When the findings already reported at 90 days were included (Swanson and Smith, 1932, b), representative blood studies were thus obtained at five intervals.

### *Results*

Reference to Table I shows that during the progress of the experiments, the concentrations of all of the constituents of the blood of normal rats increased. At the end of the 90 day period, the red blood cells had increased by 2.2 million per c.mm., they occupied a relatively larger volume, and the concentration of hemoglobin had also increased. The values for total solids and for plasma protein nitrogen at the various intervals showed that the dehydration to be expected with advancing age took place in the blood of the normal rats (Moulton, 1923). From interval to interval, the changes observed were gradual and consistent.

On the other hand, the changes that occurred in the blood of the rats on the low salt diet did not vary in the same orderly progression. There was a definite increase in the number of erythrocytes in the blood of these rats at the end of each prescribed experimental period. An augmentation took place in the first period that represented an increase of 14 per cent over the initial value in contrast to one of 10 per cent in the normal rat  $\left(\frac{d.}{p.e.d.}, 3.6\right)$ .<sup>1</sup> The influx of red blood cells was not accompanied by a proportional change in hemoglobin concentration. In the second period, erythropoiesis appears to have been particularly stimulated, for

<sup>1</sup> In the ratio,  $\frac{d.}{p.e.d.}$ , d. is the difference between means and p.e.d. is the probable error of the difference.

the number of red cells in the blood had increased markedly at 42 days, a reflection, we believe, of the failure of a normal "growth" of hemoglobin in the preceding interval. This influx of red blood cells was accompanied by an augmented concentration of pigment, in spite of which, however, a normal value was not attained. Thereafter, increments in the number of red blood cells continued at a somewhat slower rate, while the level of hemoglobin dropped

TABLE I

*Progressive Changes in Values of Certain Blood Constituents of Rats Maintained upon Normal Synthetic Ration and upon Low Salt Ration*

Experimental group	Days on experimental diet	Age of rats	No. of erythrocytes per c.mm. blood		Red blood cell volume		Hb per 100 cc. blood		Total solids		N per 100 cc. plasma	
			No. of studies		No. of studies		No. of studies		No. of studies		No. of studies	
			Average		Average		Average		Average		Average	
		days		mil-lions		per cent		gm.		per cent		gm.
Normal rats	Beginning of experiment	37	10	7.5	10	46	10	14.5	13	18.7	9	0.90
	21	58	9	8.2	6	47	8	15.5	8	19.4	6	1.04
	42	79	8	8.7	8	52	8	16.6	6	20.3	8	1.06
	63	100	11	9.0	12	51	12	16.5	10	20.2	10	1.03
	90	127	25	9.7	20	54	25	17.4	25	20.6	21	1.14
Rats on low salt diet	Beginning of experiment	37	10	7.5	10	46	13	14.5	13	18.7	9	0.90
	21	58	12	8.7	13	47	8	14.3	8	20.0	10	1.06
	42	79	13	10.7	13	49	5	15.3	5	19.4	6	1.03
	63	100	10	11.0	10	47	6	14.2	6	18.1	6	1.07
	90	127	26	12.0	22	46	25	14.3	25	19.0	27	1.06

to the initial value. The failure of the concentration of the pigment to increase concomitantly with the red blood corpuscles indicates that the quantity of hemoglobin carried by the individual cells was progressively decreasing. Calculations of corpuscular hemoglobin (Wintrobe, 1929) at the various intervals showed that this was true, whereas the absolute hemoglobin content of the normal cell decreased only slightly with advancing age. Ex-

pressed as  $x \times 10^{-12}$  gm., the corpuscular hemoglobin of the erythrocyte of the stunted rat at the various experimental intervals (beginning, 21, 42, 63, 90 days) was 19.3, 16.4, 14.3, 12.9, and 11.9, respectively. In contrast to these figures, 19.3, 19.0, 19.0, 18.3, and 18 represented the values for the corpuscular hemoglobin in the normal cell at the same stages of the experiment.

The data further show that the relative volume occupied by the red blood cells diminished in spite of the augmentation in the number of the erythrocytes. At the beginning of the period, the erythrocyte had an average volume of 61 cubic microns. Thereafter, the red corpuscles found in the blood of the rat fed the low salt diet became progressively smaller. At 21 days, the corpuscular volume was 54 cubic microns; at 42 days, 29; at 63 days, 43; and at 90 days, 37.5. On the other hand, the corpuscles in the normal rat decreased only slightly in size with advancing age, a condition that may be expected to occur as metabolic processes become less active.

Values for corpuscular hemoglobin and corpuscular volume make possible the calculation of the per cent saturation of the cells with hemoglobin (Wintrobe, 1929). At the beginning of the experiment, and after 21, 42, 63, and 90 days, respectively, the per cent of hemoglobin in the red blood cell of the normal control rats was 31, 33, 31, 32, and 32. It is of particular interest to note that the percentage of hemoglobin in the erythrocytes of the rats fed the low salt ration at the various periods is practically the same; *i.e.*, 31, 30, 30, 30, and 31 per cent. These data indicate that the per cent saturation of a cell with hemoglobin is a surprisingly constant property even in the face of a dietary adjustment particularly detrimental to pigment formation. The relative differences in the bloods of the two groups of rats are shown graphically in Fig. 1.

The picture is essentially identical with the one described by Mayerson and Laurens (1931) as characteristic of dogs sustained in an anemic condition by repeated hemorrhage for 11 weeks. Leichsenring and Hönig (1931) have also shown a progressive decrement in the size of the red blood cell in anemic dogs.

One of the first inferences from these studies might be that any set of conditions which limits the normal production of hemoglobin will induce the formation of large numbers of erythrocytes. We have shown that the first effect of the low salt ration is an immedi-

ate interference with the ability of the organism to manufacture hemoglobin. The incipient anoxemia which may result would

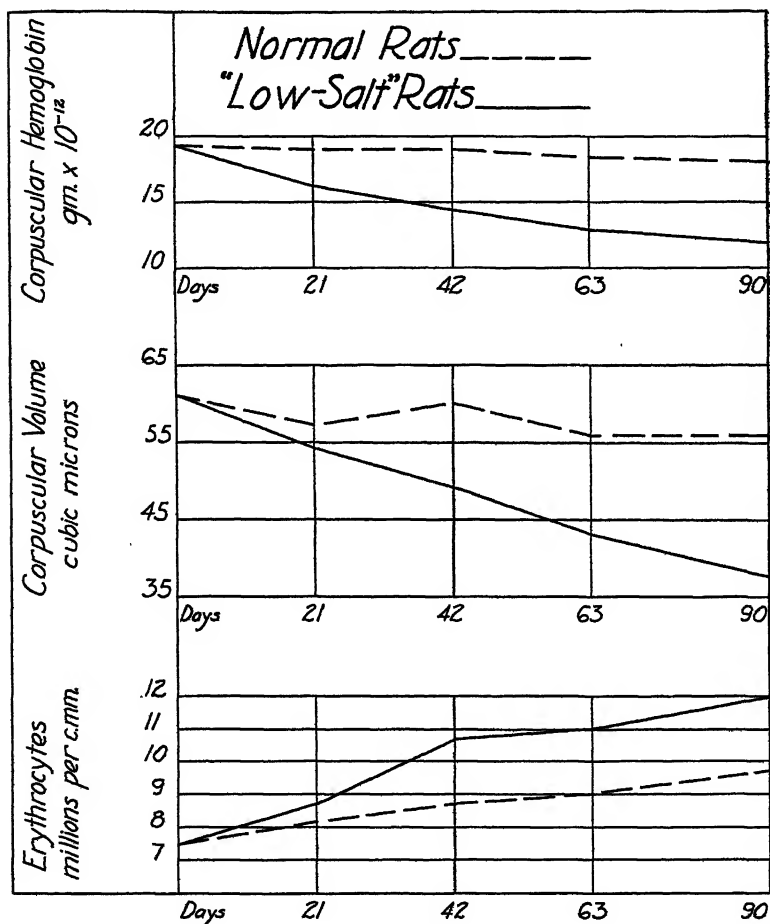


FIG. 1. The number, corpuscular volume, and corpuscular hemoglobin of erythrocytes in the blood of normal rats and of rats fed the low salt diet during a 90 day experimental period.

stimulate the formation of erythrocytes; in the present study, the average number of cells in the blood of the rat on the low salt diet at the end of 12 weeks represents an increment of 42 per cent over



the initial value in contrast to one of 16 per cent observed in the normal rats. Increased erythropoietic activity in bone marrow, the chief hematopoietic organ of the body, has been described in a state of low oxygen tension (Sabin, 1928). Campbell (1927) has also emphasized the relation between the degree of anoxemia and the subsequent secondary polycythemia that develops in animals kept at low oxygen and normal barometric pressures. Attention has been called to the fact that the greatest increments in the number of erythrocytes in the blood of the rats on the low salt diet occurred early in the experimental period and that thereafter, the rate of increase in the number of cells approached that observed in the blood of a normal rat. How long the bone marrow may be thus stimulated to produce augmented numbers of cells is questionable—not indefinitely, it appears. We have on record an animal which has been maintained on the low salt ration for 7 months. At 3 months, the red blood cell count was 10.7 million, at the end of 7 months, 3.2 million. It is surprising that life can be maintained for this length of time under the stringent dietary conditions imposed. The final reduction in the number of erythrocytes in the blood of this animal is probably a forerunner of death.

In view of the unique experimental conditions of this study, cognizance must not be lost of the possibility that perhaps the exclusion of salts from the diet interferes with the maintenance of normal respiratory exchange by disturbing the electrolyte environment to such an extent that the dissociation curve of the hemoglobin is altered (Barcroft, 1914). It may be, too, that the defective diet has induced certain qualitative changes in the chemical constitution of the hemoglobin molecule which affect its oxygen-carrying capacity. However, whether the postulated anoxemia has its origin in a change in the quantity or in the quality or in the electrolyte environment of the hemoglobin has not been determined at the present time.

As the next physiological adjustment, the organism, instead of concentrating the hemoglobin in a few large cells, forms small replicas of normal erythrocytes. Haden (1925) is of the opinion that a decrease in the size of the red blood cell is one of the best indices of drain on the bone marrow. By this mechanism, in the dearth of materials essential for the manufacture of the hemoglobin, the pigment is distributed among a greatly increased number of cells.

The resulting enormous increase in area greatly facilitates the oxygen-carrying power of the hemoglobin (Emmons, 1927; Mills, 1925).

A similar existence of a larger than normal surface area in a given volume of blood that favors a proper supply of oxygen to the tissues has recently been described in detail in individuals habituated for a lifetime to life at high altitudes (Hurtado, 1932). The mechanism of the adjustment appears to be alike in the two instances, although the etiological factor varies.

It is also possible that, under the existing conditions of scarcity of inorganic salts in the present experiment, the buffer action of certain of these is taken over in part by the hemoglobin, which, together with the transport of carbon dioxide, would be favored by the increased surface of red blood cells.

Logical as are these interpretations for the phenomena herein described, they cannot be accepted without reservation at the present time. Factors other than the postulated anoxemia may be responsible. We have evidence to show that when edestin replaces the casein in the ash-poor diet, the typical polycythemia does not occur (Swanson and Smith, 1932, *a*). The question is being studied further.

Finally, it should be noted that the hydremic condition described in the earlier paper as characteristic of the blood of the rat maintained for 90 days upon the ration poor in salts represents an early response to the diet. The only increase in the total solids of the blood occurred in the first experimental interval when nearly twice the normal increment took place. This concentration of the blood may be due to the loss of water associated with the diarrhea which followed the inauguration of the low salt régime and which was the first visible sign of any adjustment on the part of the rat to the ash-poor ration. Forster, in 1873 noted an increase in the water content of the feces of dogs receiving a ration deficient in inorganic constituents. However, the abnormally high increment in total solid concentration in this first period may be an actual reflection of the number of erythrocytes present. A relative increase in the number of red corpuscles with no change in the total blood volume will increase the total solids of the blood because the corpuscles are of higher specific gravity than the plasma (Lamson, 1915). At any rate, when the diarrheal condition disap-

peared, the blood of the rats on the low salt diet became more dilute instead of returning to a concentration normal for the age of the animal.

Changes in the concentration of the blood may be traced to various dietary inadequacies. Forster (1873) found a lower water content in the blood of dogs that had been given rations lacking in mineral constituents. His results, however, may be ascribed to the anhydremia caused by a deficiency of the vitamin B complex (see Sure, Kik, and Walker, 1929). According to Voit (1880), a deficiency in calcium caused no change in the normal concentration of total solids. Aron (1911), however, discovered an appreciable hydration of the blood following a restriction in weight induced by rations low in energy-producing constituents.

Changes in the concentration of plasma protein parallel more or less closely those observed in the total solids in both groups of rats. Evidence is conclusive that subjection to a low salt ration immediately prevents the normal processes of dehydration that occur in the blood of the rat.

#### SUMMARY

1. There is a progressive increase in the number of erythrocytes in the blood of rats maintained upon a ration containing 0.94 per cent of ash for a period of 90 days. The greatest increment occurs in the second quarter of the experimental period.

2. The maintenance of the concentration of hemoglobin at an approximately constant level for the entire experimental period may be the cause for this influx of red cells into the blood. The one small increment observed is probably associated with a particularly great increase in the number of erythrocytes found simultaneously in the blood.

3. The absolute concentration of hemoglobin in the cells progressively decreases with time.

4. The cells become progressively smaller with time.

5. Each small cell is saturated to the same degree with hemoglobin as is a normal cell.

6. After a dehydration, observed in the first quarter of the experimental period, when severe diarrhea occurred, no further concentration of the blood occurs. The blood is somewhat hydropenic at the end of the experiment.

When the rat is largely deprived of exogenous sources of the ash materials, certain alterations occur in the blood pigment and in the erythrocytes. Whether this is due to the decrement in the concentration of the hemoglobin or to certain changes in the intrinsic nature of the pigment which affect its oxygen-carrying power cannot be stated at the present time. However, with the poverty of inorganic salts in the diet, a correlation exists between the number of cells, the concentration of hemoglobin therein, and their size, which, with the resulting increase in surface area, represents the adjustment of the organism to this particular defective nutritive régime.

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# THE EFFECTS OF YEAST INGESTION ON THE COMPOSITION OF THE URINE AND FECES\*

BY H. B. PIERCE

WITH THE ASSISTANCE OF D. D. POSSON, VINCENT DU VIGNEAUD, C. A. MORRISON, Z. DU VIGNEAUD, AND M. S. PIERCE

*(From the Department of Vital Economics, The University of Rochester, Rochester, New York)*

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## INTRODUCTION

The object of this investigation has been (a) to determine the changes in the chemical composition of the urine and feces due to the ingestion of bakers' yeast and the relation of these changes to intestinal putrefaction and (b) to determine whether yeast used under controlled conditions has a laxative action. The use of yeast in the treatment of constipation is not a new or unusual practice, for Ebers' Papyrus (1), which is one of the most ancient medicopharmaceutical manuscripts, dated about 1500 B.C., recommended a combination of milk, yeast, and honey. Combe (2) has reported the work of several investigators who studied the use of yeast in the correction of alimentary disorders and intestinal putrefaction. Hawk and his coworkers (3) and Welker and Heintz (4) have found yeast to be of value in the treatment of constipation. Murlin and Mattill (5) have presented a comprehensive review of the work of a number of French investigators who used yeast successfully as a corrective for both constipation and diarrhea. Still and Koch (6, 7) have found that yeast ingestion caused a greater regularity and ease of evacuation, although the moisture content of the feces was not noticeably increased.

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Both Still and Koch (6, 7) and Thorup and Carlson (8) have observed the stools to be softer during yeast periods, due either to an increased content of water or occluded gas; however, the two latter authors have noted no appreciable effect of yeast on the alimentary rate in man or normal rats.

The question of intestinal putrefaction and the toxicity of the resultant products is discussed in all modern text-books of physiological chemistry and for more detailed information the reader is referred to these books and others by Cammidge (9), Schmidt and Strasburger (10), Combe (2), and Herter (11). Alvarez (12) has also discussed this question in his review on intestinal auto-intoxication. Putrefaction in the intestine is commonly measured by the excretion of phenols, phenol derivatives, indican, indole, and skatole, although Bergeim (13) has pointed out that we do not have any truly satisfactory chemical indices for the estimation of intestinal putrefaction. Formerly, some emphasis was placed upon the significance of ethereal sulfates as a measure of intestinal putrefaction (9, 14). However, Folin (15) and Shipley, Muldoon, and Sherwin (16) have shown that this correlation is not without qualification. Bickel (17) suggested that the volatile fatty acid content of the stool be used to determine the extent of putrefaction. But Cecchini (18) noted that volatile acids were usually present in an inverse relationship to indican. Some workers (9, 10) have found that ammonia excretion is high when marked intestinal putrefaction is noted. Folin and Denis (19) concluded that the large intestine was the chief, or at least the most constant, source of ammonia found in the portal blood, the ammonia coming from the fecal matter.

Although none of the chemical indices for determining putrefaction is entirely satisfactory, they are still used, and we have made use of them in this investigation, realizing that certain exceptions must be made in the interpretation of results.

There are very few specific references which deal with yeast ingestion and the excretion of the putrefactive products listed above. Combe (2) devoted a section of his book to a discussion of the effect of yeast on the excretion of various putrefactive products. Czernikowski (20) and Mola (21) showed that yeast ingestion caused a marked decrease in the elimination of both phenols and indole. Murlin and Mattill (5) found that when all of their

results were averaged there was a consistent, although small, relative decrease in phenols in the stools and the urine. Still and Koch (6, 7) also found a diminution in urinary phenols during periods of yeast ingestion. While Hessmann (22) found that large doses of yeast tended to cause a decreased output of indican, Murlin and Mattill (5) observed that yeast in quantities used by them had little or no effect on the excretion of this product.

The value of yeast as a protein food has been recognized for a number of years. Thomas (23) found that the protein of yeast possessed only an average value, whereas Völtz (24) and Völtz and Baudrexel (25), using both man and animal, found yeast protein to be of excellent quality. Rubner (26) and Wintz (27) concluded that a part if not all of the protein requirement of an individual could be furnished satisfactorily by yeast. From the standpoint of chemical analyses, Meisenheimer (28, 29) showed that both top and bottom yeasts contained a sufficient amount of the amino acids required by the body. Funk, Lyle, and McCaskey (30), using a dried anaerobic yeast preparation as the sole source of protein in the diet, found that the protein was poorly assimilated and had no food value. Hawk, Smith, and Holder (31) and Murlin and Mattill (5) concluded that bakers' compressed yeast formed a satisfactory article of diet for man. Still and Koch (7) found that nitrogen excretion rose during yeast periods, although the increase never amounted to the total extra nitrogen added by the ingestion of yeast.

Yeast, being rich in nucleoprotein, would be expected to have some effect upon uric acid excretion. The question naturally arose as to whether the use of yeast would result in the origin of large quantities of uric acid, and the subsequent disorders believed to be due to the presence of abnormal amounts in the body. Salomon (32) and Funk, Lyle, and McCaskey (30) reported an augmented uric acid elimination when yeast was added to the diet. Murlin and Mattill (5) observed an increase in the excretion only when three or more cakes of yeast were consumed daily, while Smith and his coworkers (33) noted no significant rise in uric acid excretion even when fifteen cakes of Fleischmann's yeast were added to the day's dietary. Still and Koch (7) found that on adding yeast to a low purine diet there was no increase in uric acid excretion, but on high protein diets there was a prompt in-



crease. As the status of this question was indefinite, special attention was paid to this fraction of urinary nitrogen.

*Criterion of Laxative Action*—Laxative action is usually associated with an increase in the frequency of bowel movements and an increase in the water content of the stool. This type of laxation is caused by the use of purgative salts which prevent absorption of water from the intestine or remove water from the circulation. Additional water in the intestinal contents induces an easier evacuation. There are other laxatives, however, which cause a more complete discharge of the bowel contents independently of their moisture content. Agar, which increases the bulk of the stool, is an agent of the latter type. Some foods and drugs cause the bowels to move earlier in the process of absorption than they would unaided. This is due to a stimulation of the intestinal wall, and the stools in this instance should contain more water, thus increasing the ease of defecation.

Yeast, then, would be considered a laxative if the moisture content of the stools and the alimentary rate were increased, or if the bulk of the stool became larger. The bulk of the stool could be increased either by the undigested yeast cells or occluded gas. It is known that yeast can remain alive in the intestine (34, 35), and with carbohydrate in the diet, fermentation occurs, the resulting gas inducing more peristalsis by distending the intestine. Again, yeast may increase the bulk of the stool by causing a greater secretion of digestive juices or by stimulating bacterial growth. It is well known that the major part of all normal stools is derived from digestive secretions (36) and that a large part of the nitrogenous matter is made up of living or dead bacteria (37). Finally, it is possible that yeast or some of its constituents have a favorable effect in stimulating the tonus of the intestinal wall (38). In this study we have interpreted laxative action as meaning an easier or more complete evacuation of the lower bowel.

*Plan of Investigation*—The subjects were four healthy adults who were engaged in laboratory work during the course of the experiments. As far as possible the work and exercise of the individuals were uniform each day.

*Diets*—Three different diets were used during this investigation. One of them, Diet I, was employed for a preliminary study with one subject. The other two diets, Diet II and Diet III, were

used with the four subjects. Diet II contained somewhat more protein, while Diet III contained more carbohydrate than was usually present in the dietary of the subjects. The food consumed was uniform in quality and quantity in each dietary régime and was prepared by an experienced dietitian in a kitchen available at the laboratory. All portions of food were accurately weighed or measured by the dietitian or by the subjects themselves before being served and in all cases the meals were consumed quantitatively. As far as possible the subjects were allowed to select the articles in the diet in order to suit their individual tastes, and the nutrients were then properly balanced.

Each diet was preceded by a preliminary period of several days in order to determine the amount of food desired and to enable the subjects to establish a more or less definite system of routine. After this preliminary period, no alterations were made in the diet, with the exception of 2 days in the last diet period when Subjects H and P were ill 1 day each.

Charcoal tablets were used for marking the feces in order to facilitate separations. Some difficulty arose in connection with these tablets because of a marked constipating effect, and their use was abandoned. It had been noted in two of the periods when charcoal had been used that no separations were necessary. No difficulty was experienced in making separations with the exception of one stool, Subject D, on the 1st day of the second control period of the last diet (high carbohydrate).

The yeast was taken in half a glass of cold water at meal times in Diets I and II, and in Diet III it was taken about 20 minutes before meals, because when taken at meal time the subjects experienced discomfort due to gas formation. The yeast was taken raw in all cases by all of the subjects, one or two cakes being taken before each meal.

Several of the subjects took 20 gm. of milk chocolate during the evening, but aside from that all food was consumed at the laboratory.

The composition of the diets with regard to each of the food principles is expressed in gm. and caloric values in Table I. The subjects did not consume excessive amounts of food in any instance as the weight of each remained constant or increased only slightly during the period of the experiment. After the second diet, all

the subjects increased the caloric intake. Whether this increase may have been due to a stimulation of the appetite by yeast is a question, for the subjects seemed to agree that they had noticed no effect of yeast on the appetite. The fact remains, however, that sizable increases in the caloric intake were made in the last diet. These increases were not due to cooler weather, because this diet started in July. No effect was observed of improved appetite during yeast periods. All of the subjects had good appetites which remained unchanged through each period, even when six cakes of yeast were consumed daily. Two subjects were nau-

TABLE I  
*Carbohydrate, Fat, and Protein in Diets, and Calories in Food Consumed\**

Subject	Diet No.	Carbohydrate	Fat	Protein	Total calories
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
H	I	330	85	69	2204
D	II	238	167	84	2876
H	II	206	108	78	2172
M	II	252	163	84	2892
P	II	225	140	76	2536
D	III	373	186	53	3476
H	III	284	165	48	2893
M	III	374	158	56	3237
P	III	433	193	57	3809

\* During the period of ingestion of three yeast cakes per day, 0.99 gm. of nitrogen (6.25 gm. of protein) was added in consequence to the diet, and during the period of ingestion of six yeast cakes per day, 2 gm. of nitrogen (12.50 gm. of protein) were added.

seated, experienced some flatulence, and were uncomfortable for several days during the last diet. One subject was ill during a yeast period, and the other during a control period. However, both subjects were able to continue the diet after a lapse of 1 day.

The first diet extended over a period of 18 days, divided as follows: first control period 5 days, a second period of 5 days during which three yeast cakes per day were added to the diet, a third period of 5 days during which six yeast cakes were added to the diet, and a fourth period of 3 days to serve again as a control. The second and third diet periods were divided into four parts of 6 days each. The first period served as a control. During the

second period three cakes of yeast per day were added to the diet. The third period served as a control, and, during the fourth period, six cakes of yeast per day were added to the diet.

Several days before going on a permanent diet, each subject delivered 24 hour urine samples at the laboratory. These were tested for albumin and sugar in order that no pathological subject might be included in the group. An attempt was made to consume the same amount of water daily, although on hot days it was necessary to consume more than the average allowance. Each subject weighed himself daily and at the same hour. Notes were made by each individual concerning his health, appetite, flatulence, number of stools passed per day, etc.

The urine and feces were collected over 24 hour periods from 8 a.m. to 8 a.m. The stools were collected in  $\frac{1}{2}$  gallon porcelain pails, were mixed and sampled immediately after delivery, and placed in the ice box as soon as the portions for analysis had been removed. The specimens for analysis were placed in glass-stoppered weighing bottles and weights obtained by difference.

The urine and feces were analyzed daily and the values in Tables II to IV inclusive are averages of five separate analyses on Diet I and six separate analyses for each period on Diets II and III.

*Methods of Analysis*—The moisture content of the feces was determined by placing samples of the well mixed stool, weighing approximately 3 gm., in small evaporating dishes and covering with ethyl alcohol. The alcohol was evaporated carefully on a steam bath and the dishes were then placed in an oven at 100° until a constant weight was obtained. Phenols in the urine and feces were determined by the methods of Folin and Denis (39, 40). Obermayer's method was used as a test for indican, the blue colors obtained in the urine specimens being compared with certain colors given on Sheet B of the color standards contained as a supplement to Mulliken's series of texts (41). The colors used were greenish blue tint 2 = 1 +, blue tint 2 = 2 +, blue tint 1 = 3 +, and blue normal tone = 4 +. Indole was determined quantitatively in the feces by use of Bergeim's method (42). The method of Folin (43) was used for the determination of inorganic and total sulfate sulfur in urine, the ethereal sulfate being obtained by difference. Neutral sulfur was not determined. Total nitrogen was determined in the urine and feces daily by the Kjeld-

dahl method, all determinations being made in duplicate. In the event that check results were not obtained, the determination was repeated the same day. 5 cc. portions of urine were used, and samples of the well mixed feces were weighed by difference from glass-stoppered weighing bottles. The method of Benedict and Franke (44) was used for uric acid determination. The titratable acidity of the urine was measured by use of the method devised by Folin ((45), p. 163). The aeration method of Folin ((45), p. 119) was used for the determination of ammonia in both urine and feces. 5 per cent suspensions of feces were prepared by rubbing up a portion of the well mixed stool with distilled water. 50 cc. of this suspension were used for each determination, caprylic alcohol being employed to prevent frothing. A trap bottle was always inserted between the aeration cylinder and the bottle containing standard acid. Air was drawn slowly through both the urine and feces samples for a period of 2 hours. A number of observations had shown that the ammonia was completely removed in this period of time. The volatile acid content of the stool was determined as follows: 400 cc. of a 5 per cent suspension of feces were transferred to an 800 cc. Kjeldahl flask, and 5 cc. of 85 per cent phosphoric acid were added. The contents were distilled with steam, 500 cc. of the distillate being collected. This volume proved to be sufficient, for only traces of volatile acids were found to be present in a second distillate of 500 cc. obtained after the initial 500 cc. An aliquot portion of the distillate was removed and titrated with 0.1 N NaOH, phenolphthalein being used as an indicator.

The reaction of the feces was determined qualitatively by placing a small quantity of the freshly mixed feces on wet litmus paper strips on glass slides. By picking up these slides and looking at the side opposite to that on which the sample had been placed, the color of the litmus could be observed.

### *Results*

*Effect of Yeast on Character and Moisture Content of Stools*—Laxative action has been discussed in the introduction of this paper, and there it was pointed out that in this investigation laxative effect has been interpreted as meaning an easier or more complete evacuation of the lower bowel. Table II presents a summary of the results of three diets. If the results of the first

TABLE II

*Weight of Stool, Moisture, Phenols, Indican, and Indole. Summary Showing Averages for 6 Day Periods*

Diet No.	Subject	Period	Weight of stool		Phenols					Indican*	Indole		
					Urinary		Fecal	Total	Total possible				
					Free	Conjugated							
			gm.	per cent	mg.	mg.	mg.	mg.	mg.	+	mg.		
I†	H	1st control	197	87.2	247	89	179	515				2.3	
		3 yeast cakes	224	86.6	258	95	211	564	575			8.2	
		6 " "	211	84.9	270	106	190	566	625			8.6	
II, high protein	D	2nd control	193	85.9	242	99	158	499				5.2	
		1st control	78	71.6	291	115	210	616		2.9		4.4	
		3 yeast cakes	106	74.6	297	115	220	633	676	3.8		8.5	
	D	2nd control	110	76.6	265	113	192	570		3.2		5.4	
		6 yeast cakes	101	73.3	296	115	186	597	726	3.6		7.0	
		H	1st control	145	83.4	311	108	285	704		2.2		7.1
	3 yeast cakes		143	81.4	329	122	290	741	764	2.6		9.0	
	2nd control		138	82.6	274	100	301	675		2.5		6.2	
	M	6 yeast cakes	157	81.8	301	139	309	750	814	2.9		15.6	
		1st control	93	77.0	170	98	78	346		0.8		3.4	
		3 yeast cakes	94	75.9	188	97	92	376	406	2.1		3.8	
	M	2nd control	84	76.9	175	97	87	359		1.8		2.5	
		6 yeast cakes	102	74.7	202	110	100	412	456	2.4		3.1	
		P	1st control	53	76.6	264	86	133	483		1.8		1.5
	3 yeast cakes		73	76.5	258	112	190	560	543	1.8		4.4	
	2nd control		54	76.9	233	99	142	474		3.0		2.6	
	III, high carbohydrate	D	6 yeast cakes	104	79.7	242	116	224	582	593	3.8		5.2
			1st control	143	80.5	286	113	215	614		2.7		4.5
3 yeast cakes			151	76.5	326	126	149	601	674	3.6		6.2	
D		2nd control	171	78.9	297	109	202	608		1.8		5.3	
		6 yeast cakes	157	77.7	297	114	173	584	724	3.8		7.1	
		H	1st control	233	85.6	274	105	345	724		1.8		6.0
3 yeast cakes			251	84.8	300	123	321	744	784	1.8		7.5	
2nd control			318	88.0	254	106	317	677		1.6		8.5	
M		6 yeast cakes	243	84.9	295	129	283	707	834	2.2		9.4	
		1st control	146	83.9	186	116	106	408		0.2		3.3	
		3 yeast cakes	186	82.9	215	115	108	438	468	1.3		4.1	
M		2nd control	158	84.6	199	128	94	421		0.7		2.5	
		6 yeast cakes	174	82.1	215	123	103	441	518	2.3		3.9	
		P	1st control	196	86.4	258	110	271	639		1.0		4.7
3 yeast cakes			334	87.9	293	136	274	703	699	2.4		8.7	
2nd control			207	85.7	248	134	209	591		0.8		4.2	
P		6 yeast cakes	219	84.7	270	118	212	600	749	2.5		8.1	

control period are used for comparison, it is seen that the weight of the stool in the majority of instances increased when yeast was added to the diet. Usually there was a greater number of bowel movements during yeast periods. The stool weights were not at all constant from day to day. The use of yeast resulted in greater regularity and ease of evacuation, the stools tending to become softer and more bulky. This was particularly noticeable during Diet II, the effects with Subjects D and P being striking. The percentages of water and dry matter were variable from day to day, during each diet, and as a general rule the moisture content of the feces did not increase when more than one stool was passed daily. In fourteen out of eighteen trials the fecal moisture was reduced during yeast periods, only four increases being noted and these with the subjects who had been constipated during control periods.

The question arises as to how the observed results may be explained. The increased weight of the stool during yeast periods may have been caused by several things; namely, greater numbers of bacteria, undigested yeast cells, secretions of the digestive tract, and undigested food residues resulting from a more rapid passage of alimentary matters through the tract. Bacterial growth may have been stimulated by yeast or its components, these bacterial cells adding to the weight of the stool. Johanson and Broadhurst (46) found that yeast added to peptone water stimulated bacterial growth markedly. References regarding this point are also given in the more recent standard bacteriological text-books. Yeast itself is not entirely digestible (35) so that living or dead cells might have increased the weight of the stool. Both Prausnitz (36) and Voit (47) found that the chemical composition of feces was fairly constant although the diet was not uniform, also that feces from fasting subjects had approximately the same composition as samples obtained after food. These results have been interpreted as indicating that the major part of excreta is derived from digestive juices. It is possible, indeed, that yeast or its components caused a greater secretion of the alimentary juices (48).

Carbohydrates in the bowel are undoubtedly fermented by yeast, and certain gases and organic acids which are known to stimulate peristalsis are formed. If fermentation were excessive, however, one would expect to find a decrease in solids rather than the increase noted.

During Diet II, the stool weights showed an average increase of 10 gm. per day during the three yeast cake period and of 22 gm. during the six yeast cake period. Similarly on Diet III the average increase in the weights of the stools was 34 gm. during the three yeast cake period and of 2 gm. during the six yeast cake period.

The weight of one yeast cake is 14 gm. and the moisture content is 73 per cent, so that during the period of ingestion of three yeast cakes daily, 42 gm. of moist or 12 gm. of dry yeast were added to the diet. During the period of ingestion of six yeast cakes, 84 gm. of moist, or 24 gm. of dry yeast were consumed. If any material quantity of yeast remained undigested and were excreted in the feces, the increased weight of the stools and dry matter would be more than accounted for. If there had been little or no digestion of yeast during the second period of yeast ingestion on Diet III, one would expect to find a greater increase in the weight of the stool and dry matter contained than was observed. Microscopic examination of the feces during yeast periods revealed the presence of many yeast cells, but only when a diarrheal stool was passed were there huge numbers present (Subject P, Diet III). On Diets I and III the greatest increase in the weight of the stools occurred during the three yeast cake period and this increased elimination extended over into the 1st days of the subsequent control period in several instances, so that indigestibility of yeast cannot alone explain the increase in fecal output after it had been added to the diet. As nearly all of the increases in the weight of the stool occurred on the first days of the yeast periods, there is evidence to indicate that the power to digest yeast may be acquired gradually.

From the results of the three series of observations, we conclude that a laxative action was existent in that softer stools having a greater bulk were passed during yeast periods. In several instances there was a pronounced effect on alimentary rate.

*Effect of Yeast on Phenol Excretion*—The formation of phenols in the intestinal tract has been discussed by Murlin and Mattill (5). Folin and Denis (40) found that catharsis caused an increase in fecal phenol excretion and a decreased elimination through the urine. They point out the fact that any tyrosine present in the feces produces a color with their phenol reagent, introducing an



error. Indole also reacts, forming a blue-colored compound. It is generally accepted that the excretion of phenols rises in gastrointestinal disorders associated with increased putrefaction. Constipation influences the phenol output to a greater extent than does diet (49). As Murlin and Mattill (5) have pointed out, the amount of phenol (and tyrosine) obtained from the stools would afford a measure of laxative effect (a) if the purgative itself did not contain tyrosine or (b) if it did contain tyrosine and all the extra quantity were recovered in the urine. The diet, of course, would have to remain constant during such an experiment. If phenol elimination diminished in yeast periods, one would conclude that putrefaction was not as great as formerly.

Large daily variations were noted in the excretion of urinary and fecal phenols on all three diets. During Diet I the quantity of phenols in both the urine and feces increased following the introduction of yeast into the diet. The total amount of phenol was not greater in the six yeast cake period than in the three. On Diet II, three of the four subjects excreted greater total amounts of phenols with yeast in the diet, the excretion by way of the feces being materially larger only with Subject P. The results obtained on Diet III show that the free phenols and usually the conjugated phenols in the urine increased during periods of yeast ingestion. Fecal phenols diminished in five of the eight trials, little change being noted in the other three during yeast periods. The total phenol excretion was usually less during the period of ingestion of six yeast cakes than during the preceding yeast period.

In general it may be stated that two bowel movements per day raised fecal phenol excretion, and the number of movements increased with yeast in twelve of the eighteen trials. It is significant that on Diet II the lowest total excretion of phenols occurred with three of the four subjects and on Diet III with two of the four subjects in the second control period. This raises the question as to whether the excretion would have been still lower during a second post-yeast period following the ingestion of six yeast cakes daily.

Little variation occurred in the values for the average percentage of conjugation of phenols during the various periods of the different diets, there being a slightly increased percentage conjugation during the yeast periods of Diet II and a slightly decreased conjugation during the corresponding periods of Diet III.

Meisenheimer (28, 29) analyzed yeast protein and found that the tyrosine content amounted to 2 per cent. With these data, the amount of tyrosine contained in the protein of the three yeast cakes would be 116 mg. and of six cakes, 232 mg. These amounts of tyrosine, if converted entirely to phenol, would yield approximately 60 and 110 mg. respectively. Determinations made in this laboratory showed the tyrosine content of yeast to be slightly higher than the values just reported. By glancing at the last column of figures under phenol excretion in Table II, it can be seen that Subject P during the three yeast cake period of each diet was the only individual who showed an increase in phenol elimination which would be in excess of that to be accounted for as arising from the tyrosine of yeast protein. If the tyrosine derived from yeast were subjected to as much putrefaction as that contained in the control diet, one would expect to find a much larger quantity of phenols in the urine, particularly during the period when six yeast cakes were added to the diet. This in itself indicates that yeast has reduced putrefaction.

Keeping in mind the fact that the smaller dose of yeast should yield approximately 60 mg. of extra phenol and the larger dose approximately 110 mg., provided the yeast protein were digested and the tyrosine converted to phenols, one finds evidence that the tyrosine fraction has been retained for some useful purpose. This statement seems more valid considering the fact that there was always a marked positive nitrogen balance during yeast periods. That the yeast protein must have been digested fairly well, is shown by the increased excretion of urinary phenols with a marked decrease in fecal phenols during Diet III. Of course there is no way of telling how much yeast has been digested in the alimentary tract, for while many of the cells have been digested, doubtless there has been a growth of new cells during the passage through the intestine.

In Diet II the greater excretion of fecal phenols during the yeast periods than during controls, indicates a laxative effect. However, there has been an increase also in the urinary phenol excretion. But, as just pointed out, this latter increase likely would have been greater if the tyrosine of the yeast protein had been subjected to the same amount of putrefaction as the tyrosine of the protein in the control diet.

Referring to Table II, we see that the total phenol elimination is practically the same for the two diets. We would expect to find a greater elimination on the high protein diet as Underhill and Simpson (49) found that the excretion of phenol and indican varied directly with the protein intake. There is a possibility that there was approximately the same amount of tyrosine in the two diets which afforded the same amount of mother substance for phenol formation, but this is to be questioned. The discrepancy is not technical, for the phenol standard was freshly prepared from the same stock solution every few days, and this stock solution did not deteriorate, because it was checked with a freshly prepared standard phenol solution at the conclusion of the third diet. As the subjects were in nitrogen equilibrium or under a condition of positive retention, it is difficult to account for the origin of phenol from circulating protein or body tissues.

*Effect of Yeast on Indican and Indole Excretion*—Indican and indole excretion have been used as a measure of intestinal putrefaction for years (15, 50), although it is recognized today that certain allowances must be made in the interpretation of results (13, 51). It is generally agreed at present that indole formation is dependent chiefly upon the amount of tryptophane in the diet (13). Sherwin and Hawk (52) found that indican in the urine fell markedly during a fast, although the average output was only slightly higher during a period of high protein intake than in a period of low protein consumption. No mention was made of fecal indole. It must be recognized that an unknown amount of indole is produced and destroyed in the body. If a series of observations extends over a long period of time, there appears to be a relationship between the quantities of indican and indole excreted.

The amount of indole in the stool varies between 0.5 and 75 mg. per day (53), and of skatole between 5 and 10 mg. Indican excretion is said to range between 4 and 20 mg. daily (9). When for any reason the food passes more rapidly than usual through the alimentary tract, the indole and skatole content of the feces is diminished (9). Lessened excretion is found in diarrhea and after the use of purgatives. Accordingly, putrefaction would be considered as being reduced and a laxative effect exerted if indole and indican excretion were lowered, provided, of course, that the agent

used contained no tryptophane. Yeast protein does contain tryptophane, and in interpreting the results, an allowance must be made for this fact.

The results of indican and indole excretion are shown in Table II. In fourteen of the sixteen observations reported there was an increase in indican elimination, no change being noted in the other two. The amount of indole in the feces was augmented in seventeen of the eighteen trials during yeast periods. There was a notable variation in indole excretion from day to day. Two bowel movements daily had no definite effect upon the amount of indole eliminated. No absolute relationship was observed between the excretion of indican and indole.

As pointed out in the introduction, certain foreign workers found that yeast ingestion caused marked reductions in the amounts of indican and indole excreted. Our results are in direct contradiction to these.

Yeast protein contains 0.5 per cent of tryptophane (28), which would mean that 29 mg. would be present in three and 58 mg. in six yeast cakes. If all of this were converted to indole, we would have approximately 16.6 and 33.2 mg. resultant from the tryptophane of the yeast protein alone. One would expect, then, to find an increased elimination of indican, indole, and skatole after yeast had been added to the diet (13). Our results in this phase of the investigation do not permit us to say that yeast decreases putrefaction, because we are not dealing with absolute quantities. If we make allowance for the indolic compounds which could arise from yeast tryptophane, it appears that putrefactive processes are limited during yeast periods.

*Effect of Yeast on Excretion of Sulfate Sulfur in Urine*—The results on sulfate sulfur excretion are presented in Table III. Large daily variations were noted in sulfate excretion during each of the three diets. The inorganic sulfates decreased during yeast periods in fifteen of the eighteen trials, while ethereal sulfates increased in seventeen of the eighteen trials. Five of the eight subjects showed a gradual diminution in total sulfate sulfur during Diets II and III. There was no apparent relationship between titratable acidity and sulfate excretion, ethereal sulfates, and conjugated phenols or between inorganic sulfate and urinary ammonia. The sulfate elimination on the high carbohydrate diet was lower

TABLE III

*Titratable Acidity, Ammonia and Sulfates. Summary Showing Averages for 6 Day Periods*

Diet No.	Subject	Period	Urine volume cc.	Titratable acid- ity, 0.1 N NaOH cc.	Ammonia			Sulfates		
					Urine mg.	Feces mg.	Total mg.	Inorganic mg.	Ethereal mg.	Total mg.
I*	H	1st control	1662			43		1203	82	1285
		3 yeast cakes	1364			118		1170	91	1261
		6 " "	1482			162		1079	125	1214
		2nd control	1497			117		1083	84	1166
II, high protein	D	1st control	1336	467	565	52	617	1824	154	1978
		3 yeast cakes	1148	453	662	71	733	1657	161	1818
		2nd control	1027	464	568	72	640	1881	124	2005
		6 yeast cakes	1262	429	592	108	700	1749	169	1918
	H	1st control	1188	325	463	88	551	1547	178	1725
		3 yeast cakes	1100	335	530	107	637	1499	187	1686
		2nd control	1169	294	478	82	560	1486	115	1601
		6 yeast cakes	1166	315	490	173	663	1381	162	1543
	M	1st control	808	454	497	43	561	1922	92	2014
		3 yeast cakes	845	458	572	67	639	1899	97	1996
		2nd control	825	436	517	49	566	1886	102	1988
		6 yeast cakes	959	443	607	77	684	1906	135	2041
	P	1st control	843	383	462	28	490	1711	127	1837
		3 yeast cakes	813	387	582	53	635	1680	137	1812
		2nd control	830	361	533	36	569	1738	133	1877
		6 yeast cakes	1083	356	523	83	606	1673	169	1842
III, high carbohy- drate	D	1st control	1052	287	342	54	396	844	124	966
		3 yeast cakes	1154	240	305	108	413	816	146	962
		2nd control	1235	198	307	97	404	914	128	1042
		6 yeast cakes	1365	219	293	131	424	993	146	1139
	H	1st control	1318	111	202	63	265	714	100	814
		3 yeast cakes	1440	98	233	103	336	744	116	860
		2nd control	1238	95	225	73	311	657	102	759
		6 yeast cakes	1492	98	210	97	307	603	100	703
	M	1st control	1043	212	235	43	278	1122	99	1221
		3 yeast cakes	973	217	266	75	341	1055	124	1179
		2nd control	861	199	202	36	238	940	106	1046
		6 yeast cakes	941	175	237	65	302	1020	114	1134
	P	1st control	1060	179	230	40	270	1000	119	1119
		3 yeast cakes	1236	202	290	94	384	1004	130	1134
		2nd control	796	197	263	39	302	947	120	1067
		6 yeast cakes	1099	159	242	96	338	929	132	1055

\* 5 day periods.

than it was during the period of greater protein intake, showing the expected dependence on this component of the diet. During none of the diets was there any uniform change in sulfate sulfur following two or more bowel movements per day.

The inorganic sulfates represent a complete loss to the body of the alkalis required for their formation. The use of yeast was followed by an increased excretion of ammonia in the urine and feces (Table III) and it is likely that this extra ammonia conserved body alkali. Yeast protein contains cystine so that if oxidation were increased or remained the same, one would expect to find an augmentation of sulfate sulfur excretion. On the other hand, there was a marked positive nitrogen balance during yeast periods so that cystine, which was formerly oxidized by the organism, may have been retained for synthesis into body protein.

The increase in ethereal sulfate excretion shows that the body was prepared to detoxicate any extra phenol or indoxyl resulting from putrefaction of yeast protein. Although the excretion of both inorganic and ethereal sulfates decreased during Diet III, the percentage reduction was much greater in the case of inorganic sulfates. The ratio of inorganic sulfate to ethereal sulfate in Diet II was greater than 10:1 in but two instances. This ratio changed materially on the diet following. Our results substantiate previous findings; namely, that ethereal sulfate is at least in part of endogenous origin. If we were to assume that a ratio of less than 10:1 were indicative of intestinal putrefaction (9), our results for the high carbohydrate diet would lead to the conclusion that there was a far greater amount of putrefaction during a diet comparatively low in its protein content.

*Effect of Yeast on Excretion of Acids and Ammonia.* (a) *On Titratable Acidity of Urine*—Although the water intake of the subjects was as nearly constant as possible, there were marked variations in daily urine volume, and the average volume for the different periods of the three diets (Table III). Urine volume was not related to either titratable acidity or ammonia, although high urinary ammonia was frequently accompanied by a high titratable acidity. The titratable acidity was always lower in the final yeast period than in the initial control period during both diets. As was expected, the acidity of the urine on Diet III was much less than on Diet II. As milk served as the chief source

of protein in Diet III, there was an abundance of calcium in the diet. The results here, as under sulfate excretion, lead to the conclusion that body alkali in the form of metallic bases has been conserved.

(b) *On Ammonia in Urine and Feces*—Reference to the various aspects of ammonia excretion is made in numerous texts. Comparatively few papers have been published which deal with fecal ammonia (9, 10). It is recognized that ammonia arises in the fecal matter contained in the large intestine (19). Fischer (54) found that stools which were alkaline to litmus contained free ammonia, and he believed that the irritating action of acids formed in the large intestine was prevented by their neutralization with ammonia.

Ehrlich (55) showed that certain amines were deaminized almost quantitatively by yeasts, alcohol and ammonia being formed. Both Dox (56) and Efront (57) demonstrated the presence of deaminases in yeast. Pierce (58) found that the addition of living yeast cells to peptone water inoculated with feces suspensions produced an increased formation of ammonia, these results also indicating the presence of an active deaminase in bakers' yeast.

The amounts of ammonia in the urine and feces are shown in Table III. The daily variations in excretion were large, particularly in the feces. Although two bowel movements *per diem* had no uniform effect on urinary ammonia, fecal ammonia rose. In eleven of the sixteen averaged tests urinary ammonia increased during yeast periods. Without exception, fecal ammonia was always higher after yeast was added to the diet, and with but one exception the same statement holds for total ammonia.

Ammonia is the most readily available alkali of the body for the neutralization of acidity. It hardly seems possible that the addition of 10 to 20 gm. of yeast protein should change the diet markedly toward the acid side; however, if ammonia and titratable acidity should parallel each other, we could infer that acid production must have been stimulated. There is evidence to show that yeast increases peristalsis, thus causing a more rapid movement of food residues and digestive juices through the alimentary tract. The drying process in the large intestine would not be so great in the cecum and ascending colon, because of a more rapid

reception of the liquid contents of the ileum. This would create more favorable conditions for bacterial action and subsequent ammonia formation. Further, the yeast deaminase would likely cause a more rapid deamination of any amino acids present, liberating additional ammonia. If there was an increased acid production, for example from the oxidation in the body of the sulfur of yeast protein, there was also a greater amount of ammonia available to neutralize this acid.

(c) *On the Reaction of Feces*—The reaction of the feces does not deviate a great deal from neutrality under ordinary conditions, although some variations are met with even in health. The litmus paper method was used to determine the reaction of the stool, but this test is not absolutely reliable as Robinson (59) showed. The test revealed that the feces became more acid during periods of yeast ingestion in spite of the fact that the ammonia content was also higher in the same periods. Since there is poor agreement between the results obtained by the litmus and the electrometric methods, it is inadvisable to discuss the results in detail.

(d) *On Volatile Acid Excretion*—Since the organic acids serve as intestinal irritants (11, 60) and thus might be partially responsible for the laxative effects frequently observed with yeast, it was thought advisable to determine the amounts excreted under the conditions of this investigation. The results are presented in Table IV. The daily variations in the excretion of volatile acids in the stools were large, amounting to over 100 cc. of 0.1 N alkali in several instances. An increased number of bowel movements was as a rule accompanied by a greater elimination of volatile acids. Fecal ammonia did not parallel volatile acid output except when there were sudden and very marked increases in ammonia excretion by way of the bowel. With the values obtained during the first control period as a basis for comparison, it is seen that in fourteen of the eighteen averaged 6 day tests volatile acid excretion was greater during yeast periods. The production of volatile acids was much higher on the carbohydrate than on the protein diet.

The marked increase in volatile acid production during yeast periods may have been due to the greater deamination of certain of the amino acids. On the other hand, there may have been more fermentation, with subsequent oxidation of the alcohols formed.



TABLE IV

*Nitrogen, Uric Acid, and Volatile Acids. Summary Showing Averages for 6 Day Periods*

Diet No.	Subject	Period	Nitrogen					Uric acid	Volatile acids in stools, 0.1 N NaOH to neutralize
			Urine	Feces	Total	In diet	Balance		
			gm.	gm.	gm.	gm.	gm.	mg.	cc.
I*	H	1st control	9.41	1.57	10.98	11.04	+0.06		24.8
		3 yeast cakes	8.80	2.05	10.85	12.03	+1.18		43.7
		6 " "	9.04	2.24	11.28	13.02	+1.74		42.9
II, high protein	D	2nd control	8.42	1.61	10.03	11.04	+1.01		36.0
		1st control	12.11	1.32	13.43	13.44	+0.01	462	74.9
		3 yeast cakes	11.38	1.73	13.11	14.43	+1.32	455	120.7
	D	2nd control	12.22	1.60	13.82	13.44	-0.38	493	95.1
		6 yeast cakes	11.72	1.65	13.37	15.42	+2.05	433	119.6
	H	1st control	10.83	1.66	12.49	12.48	-0.01	400	178.4
		3 yeast cakes	10.46	2.01	12.47	13.47	+1.00	433	174.7
		2nd control	10.12	1.68	11.80	12.48	+0.68	423	162.2
	H	6 yeast cakes	9.81	2.29	12.10	14.46	+2.36	400	238.9
	M	1st control	12.02	1.36	13.38	13.44	+0.06	350	120.6
		3 yeast cakes	12.32	1.55	13.87	14.43	+0.56	403	95.8
		2nd control	12.02	1.23	13.25	13.44	+0.19	352	92.7
	P	6 yeast cakes	12.16	1.56	13.72	15.42	+1.70	448	93.8
		1st control	11.34	0.84	12.18	12.16	-0.02	357	106.3
		3 yeast cakes	11.66	1.31	12.97	13.15	+0.18	427	97.1
	D	2nd control	11.49	0.88	12.37	12.16	-0.21	413	73.4
		6 yeast cakes	10.96	1.43	12.39	14.14	+1.75	405	126.7
III, high carbohydrate	D	1st control	6.67	1.73	8.40	8.48	+0.08	427	152.0
		3 yeast cakes	6.58	1.90	8.48	9.47	+0.99	457	170.4
		2nd control	7.07	2.27	9.34	8.48	-0.86	463	179.5
	D	6 yeast cakes	7.77	2.18	9.95	10.46	+0.51	415	165.1
	H	1st control	5.84	1.87	7.71	7.68	-0.03	390	328.4
		3 yeast cakes	6.08	2.27	8.35	8.67	+0.32	412	418.1
		2nd control	5.69	1.98	7.79	7.68	-0.11	373	446.8
	H	6 yeast cakes	5.69	2.43	8.12	9.66	+1.54	348	350.0
	M	1st control	7.46	1.47	8.93	8.96	+0.03	428	165.4
		3 yeast cakes	7.25	2.18	9.43	9.95	+0.52	443	207.5
		2nd control	6.28	1.57	7.85	8.96	+1.11	437	183.3
	P	6 yeast cakes	7.27	2.08	9.35	10.94	+1.59	392	217.7
		1st control	7.55	1.57	9.12	9.12	0.00	433	250.8
		3 yeast cakes	7.29	2.43	9.72	10.11	+0.39	480	423.8
	P	2nd control	6.75	1.80	8.55	9.12	+0.57	450	308.3
		6 yeast cakes	7.03	2.20	9.23	11.10	+1.87	432	303.7

\* 5 day periods.

In any event, it seems likely that the greater formation of organic acids with yeast in the diet may be responsible for the greater number of bowel movements in these periods. The prompt elimination of these volatile acids by the bowel and the increased ammonia production noted would in all likelihood prevent any material withdrawal of fixed alkali from the body.

*Effect of Yeast on Excretion of Nitrogen*—The favorable effects of yeast on the retention of nitrogen were discussed in the introduction. Daily variations in the amount of nitrogen in the urine and feces were over 10 per cent in numerous instances. If the results of the first control period are used for the purpose of comparison, it is seen that during periods of yeast ingestion the excretion of urinary nitrogen decreased in thirteen of the eighteen averaged 6 day trials. Fecal nitrogen, however, increased without exception in the yeast periods of the three diets, making the total nitrogen eliminated greater in thirteen of the eighteen trials. If allowance is made for the nitrogen contained in the yeast, it is seen that in the yeast periods of each diet all of the subjects were in positive nitrogen balance. In several instances more nitrogen than that to be accounted for as coming from the yeast protein was retained. With the possible exception of Subject D, Diets II and III, there is little evidence that any of this nitrogen was lost during the control periods following yeast ingestion. If the nitrogen were retained as circulating protein or in an unorganized form, one would expect to find a greater nitrogen elimination during the second control period. It is evident that the nitrogen has been organized into body protein and as such retained.

Although the nitrogen content of the stools during yeast periods increased, the extra nitrogen was not equivalent to that contained in the yeast. Several reasons for the extra nitrogen content of the feces during yeast periods may be offered. A more rapid passage of the intestinal contents would result in a lowered absorption. Bacterial growth may have been accelerated, so that some of the amino acids normally absorbed were organized into bacterial proteins, or yeast may have reproduced and organized these amino acids into yeast protein. If the secretion of digestive juices were stimulated, there would be a greater elimination of fecal nitrogen.

Damianovich (61) stated that vitamin B materially aided the synthesis of nucleins. Our results show that the excretion of uric

acid during periods of yeast ingestion did not always increase, suggesting a synthesis of nucleoprotein.

*Effect of Yeast on Excretion of Uric Acid*—The results are shown in Table IV. There was no definite relationship between urinary nitrogen and uric acid excretion on either Diet II or III. On Diet II, Subjects D and H showed no marked increase in the elimination of uric acid during yeast periods, both individuals showing more marked nitrogen retention than the other two subjects. Only one of the four subjects showed a greater excretion of uric acid during the six than during the three yeast cake period. The amount of uric acid in the urine was greater in the second control period than in the first. That delayed digestion would not serve as an entirely satisfactory explanation may be gained from the fact that the excretion during the second control period was greater during the latter days of the period. Variations in the diet and daily activity of the subject would not be sufficient to account for these changes.

The excretion of uric acid during Diet III was practically the same as during the preceding diet in which a greater amount of protein was included. This observation is of particular interest when we consider that milk was the chief source of protein in the diet. This parallelism could not be due to coffee, for Subject M had no coffee on either diet. During the six yeast cake period less uric acid was excreted than during the first control period.

Because in eight of the sixteen 6 day trials there was either a decrease or no change in uric acid excretion with yeast included in the diet, it would appear that nucleoproteins were serving to build up new tissue. Added weight is given to this statement when we consider that marked positive nitrogen balances were found during yeast periods. It is evident that individual subjects possessed varying capacities to metabolize nucleoproteins. Almost invariably an increased uric acid excretion during the first yeast period was followed by a continued high excretion in the second control period.

The results were not affected by any change in the reagents, for these were checked from time to time and at the end of the diet, a freshly prepared standard being used.

Blatherwick (62) noted that potatoes and orange juice facilitated the elimination of uric acid, and during Diet III larger

quantities of potatoes were consumed than in Diet II. This may account for the high uric content of the urine on Diet III. Harpuder and Spitz (63) noted that a dose of 10 gm. of yeast nucleic acid caused a diminution in the uric acid of the urine.

From the results obtained it does not seem probable that yeast, at least in the quantities consumed in this investigation, would cause an increase in uric acid production sufficiently great to exert a harmful effect on the organism.

#### DISCUSSION

The greater elimination of phenols, indican, and indole during yeast periods may be explained in several ways. The food proteins may be digested more rapidly or yeast enzymes may destroy sugars so that bacteria attack proteins more vigorously. It is well known that carbohydrates exert a sparing effect as regards the destruction of proteins by bacteria, as well as in the intermediary metabolism of higher animals. Bacterial growth and metabolism may be stimulated by adding bakers' yeast to pure cultures of bacteria or to feces suspensions in peptone water as shown by Castellani (64), Johanson and Broadhurst (46), and Pierce (58). If yeast ferments sugar in the intestine, as one would expect, larger quantities of CO<sub>2</sub> than usual would be formed during yeast periods, and Valley and Rettger (65) and Valley (66) have shown that CO<sub>2</sub> stimulates bacterial growth in numerous instances. The greater bacterial growth and metabolism in the presence of yeast could readily explain the greater production of phenols, indican, and indole in this investigation, however, if the tyrosine and tryptophane of the yeast protein were subjected to the same amount of putrefaction as the tyrosine and tryptophane of the proteins in the control diet.

All of the subjects were in positive nitrogen balance during yeast periods, and in several of the trials the nitrogen retained was more than equivalent to that contained in the yeast. Thus the protein available for putrefaction was not materially increased and, indeed, in certain instances was reduced.

Indole on the other hand, may limit the growth of certain bacteria. Gordon and McLeod (67) have found that indole possesses a greater antiseptic effect than phenol. Kilborn, Pierce, and Tittsler (68) have observed that indole limits the growth of certain

bacteria in culture. *Bacillus coli* has been found to be more resistant to indole than any of the other microorganisms studied. It seems likely that there may be a change in the intestinal flora during yeast periods in which greater amounts of indole are formed, in spite of the fact that Rettger, Reddish, and McAlpine (35) found no such change. It has been shown clearly by Knudson and Rose (69) that on increasing the numbers of *Bacillus coli communis* in the intestinal tract indican and ethereal sulfate formation is favored.

The decreased output of sulfate sulfur can be explained by the retention of protein by the subjects. This is exemplified by the results obtained with Subject D (Tables III and IV, Diet III) in which the first yeast period, when there was a marked retention of nitrogen, the output of sulfate sulfur decreased. During the second control period there was a negative balance, and the output rose. In the final yeast period, although the subject was in positive balance, the nitrogen retained was less than that by any of the other subjects and the sulfate sulfur showed a still further rise.

#### SUMMARY

1. Judged by the number of stools, by the weight and bulk of the stools, and by the ease of evacuation, live yeast had a laxative effect. The results were more marked in the first yeast period than in the second. The effect extended over into the following control period frequently. The action was more marked on a carbohydrate-rich than on a protein-rich diet. The moisture content of the feces as a general rule decreased during yeast periods, only constipated subjects showing an increase in the percentage of water.

2. The phenol content of the urine and feces suggests that yeast caused a diminution in putrefaction. Quantity of protein intake had no proportional effect on phenol excretion, but the proteins in different diets were not identical in kind.

3. Indican and indole excretion rose during periods of yeast ingestion. This rise was not so great as would be expected were the yeast tryptophane subjected to the same degree of putrefaction as that in the protein of the control diet.

4. The elimination of inorganic sulfates decreased and that of ethereal sulfates increased during yeast periods. Sulfate excre-

tion usually decreased after the addition of yeast to the diet. The output of ethereal sulfates was not materially influenced by diet, this suggesting endogenous origin.

5. Titratable acidity of the urine fell in the yeast periods of Diets II and III, and this, with the lowered sulfate excretion, suggests a favorable effect of yeast on acid-base balance.

6. Yeast ingestion caused a greater elimination of urinary ammonia in the majority of the averaged trials, and in every trial there was a marked rise in fecal ammonia. It is evident that the extra ammonia formed during yeast periods is for the purpose of neutralizing extra acid and, in this sense, has conserved fixed base in the body.

7. After the addition of yeast to the diet, the volatile acid excretion in the stool rose. The increase was more marked on the carbohydrate than on the protein diet.

8. Yeast nitrogen taken in amounts of three to six cakes per day was readily retained. In some instances the amount of nitrogen retained was greater than that contained in the yeast protein itself. As the nitrogen was not lost during control periods following yeast ingestion, it is likely that it had been organized into body protein.

9. In seven of the sixteen averaged 6 day trials, uric acid excretion fell or showed no change on adding yeast to the diet.

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## QUANTITATIVE STUDIES OF $\beta$ OXIDATION

### V. THE EFFECT OF INSULIN AND OF ACETOACETIC ACID ON THE PRODUCTION OF GLYCURONIC ACID\*

By ARMAND J. QUICK

WITH THE TECHNICAL ASSISTANCE OF MARY A. COOPER

*(From the Department of Surgical Research, Cornell University Medical College, New York City)*

(Received for publication, August 15, 1932)

The present enthusiasm with which glycuronic acid, galacturonic acid, and other hexuronic acids are being studied in plant products such as the pectins and gums, stands in marked contrast to the indifference with which glycuronic acid is being treated in mammalian physiology. Nevertheless, it stands to reason that any substance which the human organism can synthesize at the rate of nearly 1 gm. per hour, as the author has recently demonstrated (1), cannot be ignored without incurring the danger of overlooking perhaps an important metabolic process. In previous studies (2, 3), evidence was presented to show that glycuronic acid is derived from carbohydrates and from glycogenetic amino acids. In this paper new experiments are presented which deal more specifically with factors that influence the production of glycuronic acid. In such a study it is necessary to employ a substance which the organism will combine with glycuronic acid, and excrete promptly without further oxidation. This is essentially the same principle that was followed by Knoop in his use of phenyl-substituted aliphatic acids for the study of  $\beta$  oxidation. Many of the glycuronogenic drugs are not satisfactory, since the conjugated product undergoes oxidation. Thus, when menthol glycuronic acid is fed to a dog, only a trace appears in the urine (4).

Benzoic acid is one of the most suitable agents for studying the synthesis of glycuronic acid in the dog. It is practically non-

\* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

toxic, it is readily absorbed, and the conjugated product, glycuronic acid monobenzoate, is promptly excreted. While a small fraction of the glycuronic acid does undergo oxidation before the organism can excrete it (5), the output of glycuronic acid monobenzoate is sufficiently complete to be considered a convenient and satisfactory measure of the body's capacity to produce glycuronic acid.

In past studies it was repeatedly found that if a dog was maintained on a fixed diet, the production of glycuronic acid monobenzoate remained fairly constant even for a period of several months. It was also observed that in general, lean active dogs are apt to have a higher output of glycuronic acid than fat quiet animals.

The experimental procedure was the same as in former studies. Medium sized female dogs were employed. Only glycuronic acid was determined, since the output of hippuric acid is small and fairly constant. Sodium acetoacetate was prepared by mixing 13 cc. of ethyl acetoacetate with 100 cc. of 1 N sodium hydroxide and 400 cc. of water, allowing the solution to stand for 24 hours at room temperature, then concentrating to about 40 cc. by distillation under reduced pressure, and finally neutralizing the excess alkali.

Insulin strikingly increases the production of glycuronic acid. From Table I it can be seen that the average excretion of glycuronic acid on a standard diet after administering 5 gm. of benzoic acid is from 2.0 to 2.4 gm. for the first 6 hours. On injecting 40 units of insulin, the output is from 2.97 to 3.30 gm. which represents an increase of approximately 50 per cent. One can infer from this finding that glucose is acted upon by insulin before the organism utilizes it for the production of glycuronic acid. The question arises whether insulin activates glucose so that it unites with benzoic acid directly and then becomes oxidized to glycuronic acid, or whether insulin causes a disruption of the glucose molecule into trioses or other derivatives which the organism can utilize to synthesize glycuronic acid onto the benzoic acid molecule. No definite answer can be given until the action of insulin is known.

There are several findings, however, which suggest that glycuronic acid is probably synthesized from short chain carbohydrate

*Factors Influencing the Production of Glycuronic Acid*

Date	Dog No. and weight	Glycuronic acid excreted as glycuronic acid monobenzoate		Remarks
		6 hrs.	12 hrs.	
1928		gm.	gm.	
May 4	4 (12 kilos)	2.20	2.60	*
" 5		2.46	2.37	5 gm. sodium bicarbonate
" 7		2.38	2.60	5 " ammonium chloride
" 9		2.26	2.88	50 " glycerol
" 26		1.28	2.04	10 " acetoacetic acid. Sucrose omitted from diet
" 28		2.27		Sucrose omitted
" 30		1.87	1.97	20 gm. lactic acid
June 2		2.30	2.54	50 " glycerol
" 12		2.11	2.50	8 " uric acid. Sucrose omitted
" 14		1.60	2.24	Sucrose omitted
" 16		3.30	2.01	40 units insulin. 20 gm. extra sucrose
" 17		2.41	2.40	20 gm. extra sucrose
May 27	3 (12 kilos)	2.36	2.38	
June 2		1.68	0.45	25 " lactic acid
" 4		2.84	2.44	
" 7		1.38		20 " glycolic acid
" 13		2.22	2.45	8 " uric acid. Sucrose omitted
" 16		2.04	2.05	Sucrose omitted
" 17		3.14	2.46	40 units insulin. 20 gm. extra sucrose
" 19		2.38	1.60	20 gm. extra sucrose
" 21		2.94	1.94	15 " alanine
" 5	6 (10 kilos)	1.93	2.34	
" 7		1.80	2.36	10 " acetoacetic acid
" 11		1.43	2.43	10 " " " Sucrose omitted
" 14		2.33		
" 17		2.92	1.59	40 units insulin. 20 gm. extra sucrose. Dog vomited
" 19		2.44	1.88	20 gm. extra sucrose

\* 5 gm. of benzoic acid as the sodium salt were fed in each case with a diet consisting of 60 gm. of casein, 40 gm. of lard, 2 gm. of bone ash, and 50 gm. of sucrose (unless otherwise noted). All acids were neutralized

derivatives. It will be recalled that the writer (3) found that the completely diabetic dog can utilize the glycogenetic fraction of the protein molecules, which would otherwise be lost as urinary sugar, for the production of glycuronic acid. Since it is generally assumed that the glycogenetic amino acids yield one or more simple derivatives from which the organism can synthesize glucose, it seems logical that the body may use these same derivatives to produce glycuronic acid. It is interesting to find that 15 gm. of alanine markedly increased the output of glycuronic acid. From these preliminary results one is inclined to conclude that glycuronic acid is synthesized from glucose only after the latter has been acted upon by insulin, but that it is derived directly from the glycogenetic amino acids.

A fairly pronounced decrease in the excretion of glycuronic acid occurs after feeding acetoacetic acid. This suggests that acetoacetic acid either competes for the same substance which the organism uses for the production of glycuronic acid, or it exerts an inhibitory influence on the mechanism which synthesizes or conjugates glycuronic acid. The first explanation seems the more probable. Recently the writer (6) proposed the hypothesis that fatty acids may perhaps in some stage of their catabolism become conjugated with glycuronic acid in a manner similar to the union of the latter with benzoic acid and phenylacetic acid. While the present observation does not furnish direct proof for this hypothesis, it must be admitted that it is rather significant that acetoacetic acid presumably requires for its catabolism a substance which is also needed in the synthesis of glycuronic acid.

Lactic acid, as well as glycolic acid, decreases the output of glycuronic acid. This observation is interesting in view of the fact that these acids also decrease the excretion of uric acid, whereas carbohydrates or rather an antiketogenic derivative appears to stimulate the excretion (7). It seems advisable, however, to defer any explanation for the action of lactic acid until more information concerning its metabolism is available. It is obvious, however, that lactic acid is not the precursor of glycuronic acid. Although the excretion of uric acid is influenced by carbohydrate metabolism, the feeding of uric acid does not appear to have any effect on the synthesis of glycuronic acid.

All attempts to determine the effect of pyruvic acid on the production of glycuronic acid failed, for the dog invariably vomited after receiving this substance. Unfortunately, the effect of methylglyoxal, dihydroxyacetone, and various amino acids was not determined. Mild alkalosis and acidosis, such as is produced by sodium bicarbonate and ammonium chloride respectively, do not seem to influence the synthesis of glycuronic acid.

#### SUMMARY

The excretion of glycuronic acid monobenzoate following the administration of benzoic acid to a dog has been employed as a measure of the organism's capacity to synthesize glycuronic acid. It was found that insulin definitely stimulates the production of glycuronic acid, while the feeding of acetoacetic acid, lactic acid, and glycolic acid decreases the output.

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## THE DIGESTION AND INACTIVATION OF CRYSTALLINE UREASE BY PEPSIN AND BY PAPAIN

By JAMES B. SUMNER, J. STANLEY KIRK, AND STACEY F. HOWELL

*(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca)*

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The identity of the enzyme urease with the octahedral globulin crystals isolated by the senior author from the jack bean in 1926 (1) has been challenged by Waldschmidt-Leitz and Steigerwaldt (2). These investigators published a paper in which they claimed that crystalline urease is not inactivated at pH 7.0 by incubation with trypsin or papain, while the protein component of the urease undergoes digestion to such an extent that after 48 hours at 30° no more than a hardly perceptible precipitate is given upon adding sulfosalicylic acid.

We have shown (3) that the experimental work of Waldschmidt-Leitz and Steigerwaldt is not to be accepted since their digests contained only 1 part of crystalline urease in 67,000, at which dilution urease gives a hardly perceptible precipitate with sulfosalicylic acid, and since the precipitate which is given by proteins present in the trypsin itself is so heavy as to interfere and to prevent one from drawing any conclusion concerning the amount of precipitate given by the urease.

We have carried out digestions in which the urease precipitable with sulfosalicylic acid has been determined gravimetrically both before and after incubation with trypsin and have shown that no significant amount of digestion could have occurred. Finally, by making use of the delicate reaction between urease and antiurease, we have demonstrated that during incubation with trypsin the decrease of the protein component of urease proceeds at the same rate as diminution of urease activity.

In reply to our paper Waldschmidt-Leitz and Steigerwaldt (4)



state that our precipitin test for urease, with immune rabbit serum, cannot be considered to be a test for the protein of the urease but only a test for the enzyme itself. They have carried out more digestions over very long periods of time, using purified trypsin and larger amounts of urease. They claim to be able to show by nephelometric means that the protein diminishes more rapidly than the urease activity. Hence they again declare that our urease crystals are not identical with the enzyme, but act simply as a favored carrier.

We emphatically disagree with the statement that our precipitin test with antiurease is not a test for the protein. We have attempted to purify tryptase as briefly described by Waldschmidt-Leitz and Steigerwaldt by adsorbing on kaolin at pH 5.0, extracting with  $M/15$  phosphate buffer at 5.0, and precipitating with 80 per cent alcohol, and have not succeeded. We were unable to extract more than a trace of tryptase at pH 5.0 and we found that precipitation with 80 per cent alcohol caused considerable inactivation. Therefore, until we can obtain a suitable trypsin preparation we shall not be able to repeat the work of Waldschmidt-Leitz and Steigerwaldt. However, we have found that pepsin and also papain- $H_2S$  rapidly inactivate crystalline urease at pH 4.3 and that hydrolysis of the urease proceeds at practically the same rate as the inactivation (see Fig. 1). Since, therefore, hydrolysis of urease by pepsin and papain inactivates urease, it is extremely unlikely that urease can be hydrolyzed by trypsin without inactivation, as claimed by Waldschmidt-Leitz and Steigerwaldt. Even if such an anomalous difference of attack by trypsin were possible, nevertheless this discovery could not be used as an argument that urease is not a protein.

While trypsin interferes with attempts to follow urease digestion because it gives a precipitate itself with sulfosalicylic acid, we have found that pepsin (Parke Davis 1:10,000) remains water-clear after addition of sulfosalicylic acid. Hence it is possible to follow the hydrolysis of urease in parallel with its inactivation by measuring the amount of precipitable material. However, urease precipitated by sulfosalicylic acid is rapidly digested by pepsin. But dilute pepsin solutions give no precipitate with dinitrosalicylic acid, whereas this reagent prevents pepsin from attacking the precipitated urease. Furthermore, while urease gives a moder-

ate precipitate when added to the buffer at pH 3.9 to 4.9, in the presence of pepsin it forms a heavy precipitate and the solution of this precipitate has been found to coincide with the inactivation of the urease.

In our earlier experiments we employed rather concentrated pepsin solutions with the idea that the more concentrated the pepsin the more rapid would be the rate of digestion of urease.

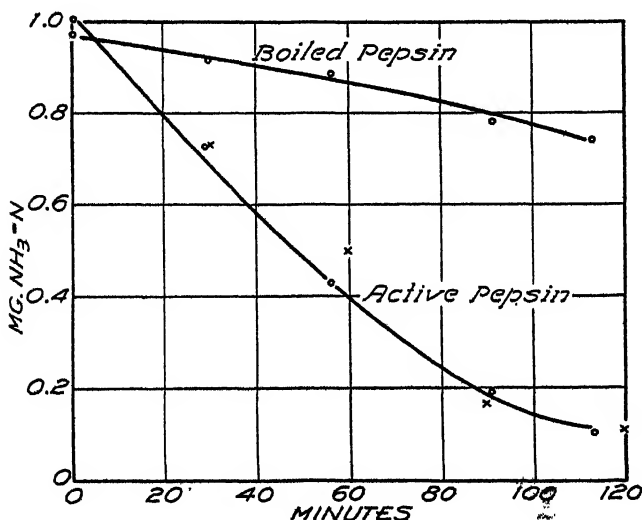


FIG. 1. Urease digested by pepsin (40 mg.) at 38° and pH 4.3. × indicates relative turbidity with dinitrosalicylic acid. For *K* values see Experiment 8, Table I.

However, further work has shown that concentrated pepsin solutions actually prevent digestion and that the optimum effect is obtained when one treats urease with somewhat more than its own weight of pepsin (see Fig. 2). In this connection we are inclined to wonder whether Waldschmidt-Leitz and Steigerwaldt have not employed more trypsin in their digestions than really necessary.

It might be expected *a priori* that at pH 4.3 the spontaneous inactivation of urease would be so rapid as to make it difficult to observe the inactivation caused by peptic digestion. This, however, is not true, for under optimum conditions the inactiva-

tion by pepsin has been found to be from 10 to 20 times more rapid than inactivation by acid alone (see Fig. 2 and Experiments 3 and 11, Table I). In solutions more acid than pH 4.3 inactivation by pepsin becomes continually more rapid (see Experiments 12 and 13, Table I), but with decreased acidity the effect of pepsin diminishes and at pH 4.7 it is very slight (see Experiment 1, Table I). We found urease to be rapidly inactivated by as little

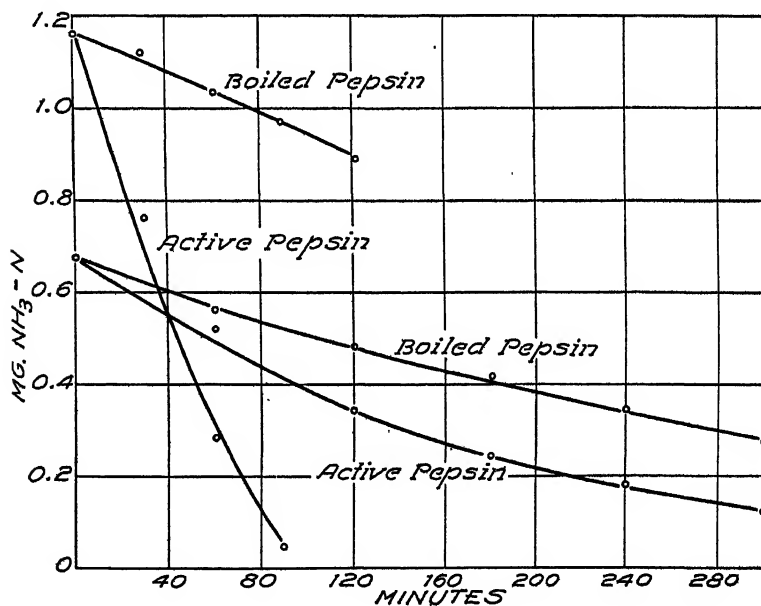


FIG. 2. The upper curves represent boiled and active pepsin (8 mg.) at 38° and pH 4.32. The lower curves represent 200 mg. of pepsin at 38° and pH 4.3. For K values see Experiments 3 and 4, Table I.

as 1.6 mg. of pepsin (see Fig. 3). When we incubated recrystallized urease with crystalline pepsin the rate of inactivation was fully as rapid (see Fig. 4).

Zakowski (5), using impure jack bean urease, found inactivation with papain activated by HCN, H<sub>2</sub>S, and cysteine at pH 4.4 to 5.5. Our work reveals that papain-H<sub>2</sub>S readily digests and inactivates urease at pH 4.2, but that at pH 4.8 it has hardly any action (see Fig. 5 and Experiments 14 to 16, Table I).

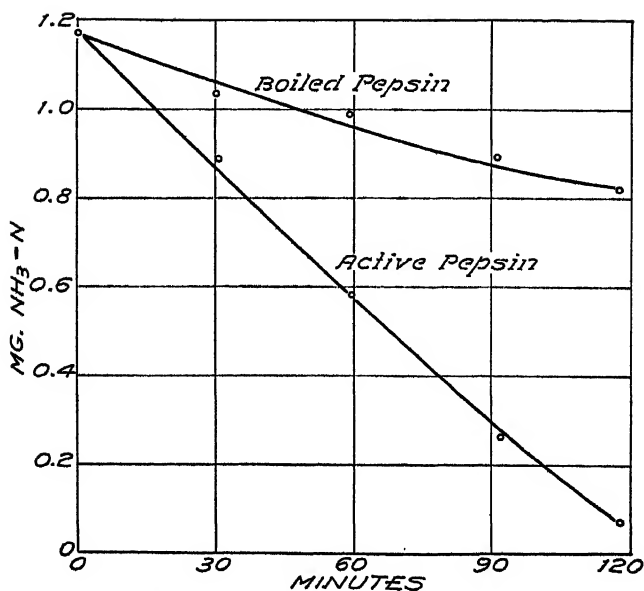


FIG. 3. Urease digested by pepsin (1.6 mg.) at  $38^\circ$  and pH 4.3. For values of  $K$  see Experiment 7, Table I.

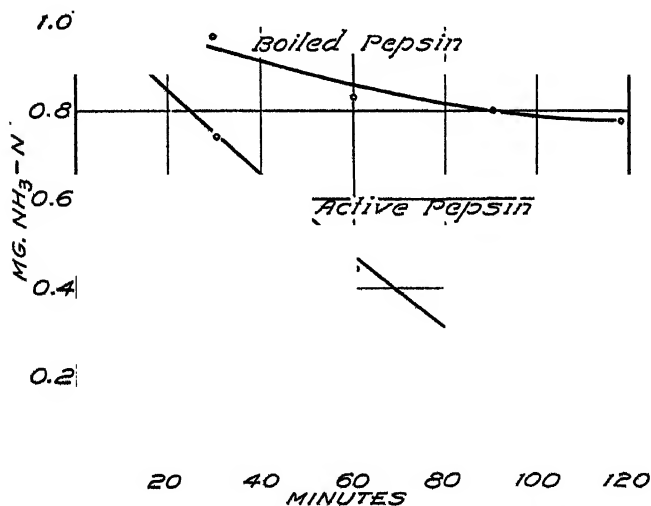


FIG. 4. Recrystallized urease digested by crystalline pepsin (3 mg.) at  $38^\circ$  and pH 4.3. For  $K$  values see Experiment 9, Table I.

TABLE I

*Effect of Proteolysis, pH, and Temperature upon Inactivation of Urease*

Experiment No.	Temperature	pH	Enzyme		K active	K boiled
	°C.			mg.		
1	38	4.7	Pepsin	8	0.00038 0.00071 0.00104	0.00006 0.00008 0.00015
2	38	4.54	"	200	0.00054 0.00057 0.00067	0.00034 0.00030 0.00026
3	38	4.32	"	8	0.0064 0.0102 0.0152	0.00052 0.00087 0.00086
4	38	4.3	"	200	0.0018 0.0024 0.0024 0.0024 0.0024	0.0013 0.0012 0.0012 0.0012 0.0012
5	38	4.3	"	200	0.0025 0.0025 0.0026 0.0027 0.0028 0.0027	0.0007 0.0011 0.0013 0.0015 0.0014 0.0016
6	38	4.3	"	40	0.0068 0.0088 0.0092	0.0011 0.0011 0.0011
7	38	4.3	"	1.6	0.0039 0.0051 0.0070 0.0105	0.0011 0.0011 0.0011 0.0012
8	38	4.3	"	40	0.0049 0.0066 0.0079 0.0085	0.00091 0.00085 0.00099 0.00099
9	38	4.3	Crystalline pepsin	3	0.0057 0.0048 0.0048 0.0061 0.0067	0.0012 0.0012 0.0009 0.0012 0.0012
10	0	4.2	Pepsin	200	0.0092 0.00049 0.00050 0.00062 0.00056	0.0010 0.00048 0.00038 0.00040 0.00040

TABLE I—*Concluded*

Experiment No.	Temperature	pH	Enzyme		K active	K boiled
	°C.			mg.		
11	48	4.3	Pepsin	8	0.019	0.0010
					0.033	0.0020
					0.055	0.0023
12	38	4.0	"	200	0.054	0.025
					0.068	0.024
					0.074	0.024
13	38	4.0	"	200	0.054	0.024
					0.053	0.024
						0.024
14	38	Ca. 4.8	Papain-H <sub>2</sub> S	3.3	0.00082	0.00052
15	38	" 4.2	"	3.3	0.0024	0.00052
					0.0026	0.00077
					0.0032	0.00090
					0.0034	0.00094
					0.0036	0.00112
16	38	" 4.2	"	16.5	0.0020	0.0015
					0.0025	0.0015
					0.0028	0.0015
					0.0028	0.0016
					0.0028	0.0015

We have evidence that the temperature coefficient for peptic inactivation of urease is greater than the coefficient for inactivation of urease by acid. At 0° the difference between inactivation by boiled and by active pepsin is slight (see Experiment 10, Table I), whereas at 38° it is considerable (see Experiments 3 to 9), and at 48° it is still greater (see Experiment 11).

In determining the activity of urease that has been kept at pH 4 to 5 we have found it necessary to observe especial precautions, for in acid solution urease undergoes two kinds of inactivation, reversible and irreversible. The reversible inactivation is rapid. Upon bringing acidified urease to neutrality reactivation reaches a maximum value within about 20 minutes. Hence, after withdrawing samples from our digests these have been pipetted into excess of neutral phosphate-gum arabic solution and have been allowed to stand for at least  $\frac{1}{2}$  hour before analyzing for urease activity.

The irreversible inactivation of urease by acid has been found to

agree rather well for the equation for a monomolecular reaction. With the equation,  $K = \frac{1}{t} \log_{10} \frac{A}{A-X}$ , where  $t$  = minutes of incubation,  $A$  = activity of urease at the start, and  $A - X$  = activity after  $t$  minutes, we have calculated the values of  $K$  at different time intervals both for urease treated with boiled pepsin and papain and for urease digesting with active pepsin and pa-

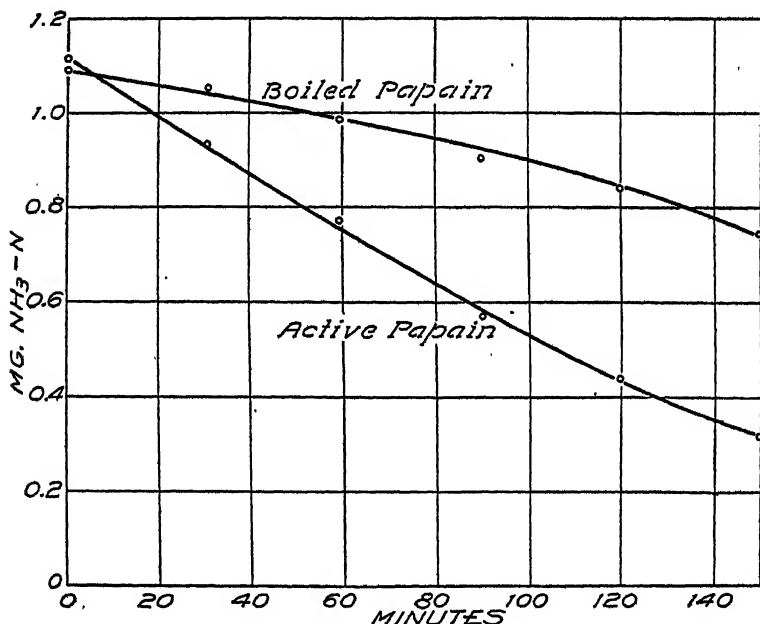


FIG. 5. Urease digested by papain-H<sub>2</sub>S (3.3 mg.) at pH about 4.2 and 38°. For  $K$  values see Experiment 15, Table I.

pain. With excess of pepsin the values are constant, while with small amounts of pepsin and papain they increase with time. Nevertheless these values are given in Table I, since they show concisely the differences in rates of inactivation of urease under different conditions.

#### EXPERIMENTAL

We have employed Parke Davis' pepsin 1:10,000, Northrop's crystalline pepsin (6), and Boehringer's papain 1:350. The papain was activated by incubating 10 cc. with 10 cc. of water saturated

with hydrogen sulfide for 35 minutes at 38°. In each experiment 20 cc. of pepsin or activated papain were used. Our controls were exactly the same as our digestion experiments, except that the enzyme employed had been heated in boiling water to inactivate. The pepsin was heated 10 minutes, the papain 20 minutes.

In each experiment we have employed 2 cc. of acetate buffer made by mixing N acetic acid with N sodium acetate. The 2 cc. of buffer were added to the controls only after these had been heated and cooled to room temperature. Determinations of the pH values of peptic digests were made by quinhydrone electrode both at the beginning and the end of each experiment and no significant differences were observed either with lapse of time or with reference to boiled and active solutions. It was not possible to employ the quinhydrone electrode with the papain-H<sub>2</sub>S digests. Here we have determined the pH colorimetrically, using brom-cresol green.

1 cc. of crystalline urease was used in each experiment, making the total volume 23 cc. The urease amounted to 400 to 800 units, or from 3 to 6 mg. In Experiment 9 the urease had been once recrystallized from 30 per cent alcohol. For determination of urease activity 1 cc. samples of the digests were pipetted into 40 cc. of neutral phosphate-gum arabic solution. This solution was prepared by heating 40 gm. of powdered gum arabic in water to dissolve, then adding 150 cc. of 9.6 per cent neutral phosphate, filtering, and diluting to 2 liters. To 300 cc. of this solution were added 150 cc. of 9.6 per cent neutral phosphate. Water redistilled from glass was used for all solutions.

Determinations of urease activity were made as usual except that they were run at 30°. Where most of the urease had been digested, the runs were for longer periods of time than the usual 5 minutes. Our results are expressed as mg. of NH<sub>3</sub>-N per 5 minutes.

Determination of undigested urease was made by adding 10 cc. of 1.3 per cent dinitrosalicylic acid to 2 cc. of digest and comparing the turbidity with a set of standards containing known amounts of urease precipitated by dinitrosalicylic acid.

#### SUMMARY

Crystalline urease is rapidly inactivated by pepsin and by papain-H<sub>2</sub>S and this inactivation coincides with the proteolysis.



At pH values more acid than 4.3 the rate of inactivation is increased, while at values less acid the rate is decreased. Lowering the temperature to 0° greatly slows the rate of inactivation. The inactivation of urease by acid obeys the monomolecular law. As result of evidence brought forth in this paper the claim of Waldschmidt-Leitz and Steigerwaldt that crystalline urease cannot be considered identical with the enzyme proper can no longer be maintained.

We wish to acknowledge our appreciation to both the Heckscher and the Sage Foundations for financial assistance in this research.

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# INFLUENCE OF CERTAIN IONS UPON THE EXTRACTION OF MALT AMYLASE FROM ALUMINA GEL BY WHICH IT HAS BEEN ADSORBED

BY M. L. CALDWELL AND S. E. DOEBBELING

*(From the Department of Chemistry, Columbia University, New York)*

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In the course of experiments dealing with the purification of malt amylase, certain hitherto unrecorded properties of this enzyme were encountered in connection with its adsorption by alumina gel. These have been quantitatively studied and the results are reported briefly here both because of their theoretical interest and of their practical importance to others engaged in similar work.

The researches of Willstätter and his collaborators (1) have resulted in renewed interest in the general subject of the purification or concentration of enzymes and especially in the use of adsorbing agents for this purpose. The general principle of such work is to use an adsorbing agent either to remove the enzyme from its solution, leaving many accompanying impurities unadsorbed or, in some cases, to adsorb away inactive material leaving the enzyme in solution with fewer impurities. When the enzyme is removed from a solution by adsorption, it is usually recovered from the adsorbing agent by extraction with some suitable solvent.

Adsorption of the amylase by alumina gel was found useful by Lüers and Sellner (2) in their method for the purification of malt amylase and is also an important step in the method recently developed in this Laboratory (3) for the purification of pancreatic amylase.

The present report deals with the recovery of malt amylase from alumina gel by which it had been adsorbed and the influence of certain ions upon this. The details and results of the purification procedure as a whole will not be discussed here.

In the work above referred to (3), it was found possible to ex-

tract pancreatic amylase readily from the alumina gel by the use of phosphate solutions (0.01 M) so adjusted that they gave a final pH of about 7.1 or by the use of dilute sodium hydroxide solutions (0.015 M) which, under the conditions used, gave approximately the same hydrogen ion activity in the extracted solutions. The hydrogen ion activity of the solution used to extract pancreatic amylase from the gel was found to be a very important factor both in the extraction of this enzyme from the gel and in its stability in solution. In order to minimize the contamination of subsequent precipitates with salts, sodium hydroxide solutions of suitable hydrogen ion activity were chosen for this step in the method for the purification of pancreatic amylase. After the extraction, the active enzyme was immediately precipitated by alcohol and ether and solid products of extremely high enzyme activity were readily obtained (3).

In the purification of malt amylase, Lüers and Sellner (2) used phosphate solutions of about pH 7.4 to extract the enzyme from the alumina gel by which it had been adsorbed. This we also found feasible but attempts to use sodium hydroxide solutions of the same final hydrogen ion activity instead of phosphate solutions as had been possible with pancreatic amylase gave unsatisfactory results.

It soon became evident that the hydrogen ion activity of the solution was not the only factor involved in the extraction of malt amylase from its "activated" alumina gel. The use of sodium hydroxide solutions of 0.015 to 0.03 M, which caused the solutions after the extraction of the activated gel to differ at intervals between pH 6.5 and 11.6, failed to bring about a measurable extraction of the amylase which had been partially purified before the adsorption. When sodium hydroxide solutions which gave a final pH of 6.5 to 7.4 were used, the amylase activity was found almost quantitatively in the gel after the extraction and it could be partially removed by the subsequent use of phosphate solutions with a final pH of 7.1. With sodium hydroxide solutions of a final pH of 9.3 and with more alkaline solutions, no measurable enzyme activity remained either in the gel or in the solutions centrifuged from it.

The difference in the results obtained with phosphate and with sodium hydroxide solutions of the same final hydrogen ion activity

and the recent work of Thomas and his collaborators (4, 5) suggested the possibility that interchange of ions might be an important factor in the extraction of the active amylase from the alumina gel by which it had been adsorbed. The results of quantitatively comparable measurements with solutions of different electrolytes indicate that this hypothesis is probably correct; the ions present in the extracting solution have a very marked influence upon the results obtained.

#### EXPERIMENTAL

The aqueous suspension of alumina gel used in these experiments was prepared (3, 6) as follows: Approximately 0.6 M aluminum sulfate was poured into an excess of 15 per cent ammonia solution. Steam was led through the alumina suspension thus formed for 10 to 12 hours. The gel was then washed by decantation until it no longer settled upon standing and until it was free from sulfate. The  $\text{Al}_2\text{O}_3$  content of aliquot samples of the suspension was determined by evaporation to dryness and ignition to constant weight.

For use, the alumina gel suspension was made up to contain 0.5 gm. of  $\text{Al}_2\text{O}_3$  per 100 cc. and was buffered to pH 4.8 to 5.0 by means of acetic acid and sodium acetate which were present in a total concentration of 0.01 M acetate.

Alumina gel was also prepared without heat treatment from aluminum sulfate and from aluminum chloride. Such gels were unsatisfactory for the purpose of these experiments because of their formation with the enzyme solutions of pasty suspensions which did not respond to centrifuging. As the gel prepared from aluminum sulfate, as outlined above, was found to adsorb the amylase almost quantitatively from most of its solutions, it was considered sufficiently reactive for this work.

The procedure by which direct comparisons were made was as follows: Equal volumes of the same enzyme solution were treated with equal volumes of the same buffered alumina gel suspension. After being mechanically stirred for 30 minutes, the mixtures were centrifuged and the supernatant liquid removed. The activated alumina gel in each container was immediately stirred up with an equal volume of one of the solutions being studied. These mixtures were then mechanically stirred for 2 hours, centrifuged, and the solution and solid each tested for enzyme activity.

The mechanical stirring was comparable as the containers were of the same size and shape (250 cc. conical centrifuge cups) and similar glass stirring paddles were attached to pulleys of equal size connected to the same motor.

On account of the instability of the enzyme, all solutions were kept at 0° by the use of melting ice baths.

The conditions adopted for the adsorption of the enzyme and the time intervals allowed for its adsorption by the alumina gel and for its extraction from the gel were those which had previously been found to yield the most satisfactory results.

The enzyme activity of the solutions and of suspensions of solids was measured quantitatively by gravimetric determination (7) of the reducing sugar (mainly maltose (8)) formed when a given volume of the solution or suspension being examined was allowed to act at 40° ( $\pm 0.01^\circ$ ) for 30 minutes upon a suitably prepared starch substrate (9). All data were corrected for reducing action of starch and reagents.

The salts studied for their effectiveness in extracting malt amylase from the alumina gel by which it had been adsorbed were purified by recrystallization. The results obtained with them were in several cases confirmed by the use of solutions prepared by mixing stoichiometrical proportions of the acid and base concerned. The purity of the sodium chloride and of the sodium phosphates was confirmed by spectrographic examination with graphite electrodes.<sup>1</sup>

None of the salts reported was found, in the concentrations used, to influence the activity of the enzyme or the formation of reducing sugar from the starch. Any differences observed in the enzyme activity of the solutions were therefore presumably due to different concentrations of enzyme present.

While of interest theoretically, it was not feasible to include potassium ferri- or ferrocyanide in this comparison as they interfered with the measurement of enzyme activity.

Reproducibility of results was demonstrated not only by repeated comparisons of the influence of the different salts but by repeated direct comparisons of the results obtained with the same salt. This is illustrated by the following typical data. 8 equal

<sup>1</sup> We wish to thank Dr. Andrew Dingwall for making these spectrographic examinations.

volumes of an enzyme solution were treated, as described above, for adsorption by equal volumes of the same buffered alumina gel suspension. After centrifuging, four of the activated solids were extracted with equal volumes of 0.10 M sodium potassium tartrate solution while four were extracted with equal volumes of 0.10 M disodium tartrate solution. After the extraction, the suspensions were centrifuged and the solutions measured for enzyme activity. This, expressed as mg. of maltose per 0.10 cc., was found to be 211, 211, 210, and 208 respectively for the four sodium potassium tartrate extracts and 125, 125, 127, and 125 respectively for the four disodium tartrate extracts. In an entirely separate experiment, the following results were again obtained with 0.10 M disodium tartrate solution: 163, 165, 162, and 165 mg. of maltose per 0.10 cc. of the extract. These and similar results made it evident that the method was reliable.

The differences in the effectiveness with which the salts extract malt amylase from the activated alumina gel were confirmed by repeated experiments with the same and similar solutions of malt amylase and with solutions of the enzyme at different stages in its purification or concentration. While the absolute values with enzyme solutions of different degrees of purification differ, the relative results are in good agreement. This indicates that the observed differences depend upon properties of the enzyme itself and not solely upon impurities which accompany it in solution or during the adsorption process.

All of the salts reported were studied in concentrations of 0.10 and 0.01 M. Some were also studied in concentrations of 0.05 M. Concentrations higher than 0.10 M were not investigated as they were found for several of the salts to influence the activity of the enzyme.

As would be expected from our knowledge of aluminum hydroxide (4, 5, 10), treatment of the activated alumina gel under strictly comparable conditions with the different salt solutions or with different concentrations of the same salt resulted in different final hydrogen ion activities in the centrifuged extracting salt solutions. This is in accord with the recent findings of Thomas and coworkers (4, 5) who explain such results in terms of the penetration of the anions of the salts into the complex aluminum ionic micelles with a resulting displacement of hydroxo or of

other coordinately bound groups. It has been pointed out that the hydrogen ion activities obtained in such cases depend upon many interrelated factors of which one is the nature of the penetrating ion.

In the present work a very marked relation has been found between the salt used and the extraction of malt amylase from its activated alumina gel but no consistent correlation has been observed between this action and the final hydrogen ion activity of the centrifuged extracting solution. In general, the results indicate better extraction of the amylase when the neutral salt solution is used as such without any attempt to adjust its hydrogen ion activity than when this has been adjusted. This is indicated typically by the data for 0.10 M citrates given in Table I. Here the solutions having an original pH of 6.4 and 8.4 represent the

TABLE I

*Influence of Hydrogen Ion Activity upon Extraction of Malt Amylase from Alumina Gel by Potassium Citrate Mixtures (0.10 M with Respect to Citrate Ion)*

Original pH.....	11.8	5.8	6.4	8.4
Final pH.....	8.6	5.8	7.0	7.2
Amylase activity, mg. maltose per 0.1 cc.....	221	220	309	304

extremes of the stoichiometric part of the titration curve as experimentally determined. With these solutions, approximately the same final pH was obtained after centrifuging, and equal extraction of the amylase was effected. The solutions with an original pH of 5.8 and 11.8 respectively represent the more acid and more alkaline parts of the titration curve, beyond its stoichiometric portion. These solutions resulted in different final pH values in the centrifuged solutions and the extraction of the amylase was lower in both cases.

Quantitative comparisons of the influence of the different salts upon the extraction of malt amylase from the alumina gel by which it had been adsorbed are given in Tables II and III. These data are typical of the relative results repeatedly obtained with these salts and in each case are strictly comparable as the measurements were made side by side starting with the same enzyme solu-

tion and the same alumina gel suspension and keeping as constant as possible all factors other than those introduced by the use of the different salts.

It is evident from the data given that the influence of the different salts in this connection is very different; the phosphates and the citrates of sodium and potassium are much more efficient in the

TABLE II

*Influence of Ions upon Extraction of Malt Amylase from Alumina Gel by Which It Had Been Adsorbed from Unpurified Malt Extract (Power Approximately 30)*

Solution	Concentration	Original pH	Final pH	Enzyme activity Maltose per 0.1 cc.
	<i>mols per l.</i>			<i>mg.</i>
Dipotassium phosphate*.....	0.10	8.8	7.8	636
Tripotassium citrate*.....	0.10	7.4	6.9	576
Disodium phosphate*.....	0.10	8.8	7.4	573
Trisodium citrate*.....	0.10	7.6	6.9	421
Sodium potassium tartrate.....	0.10	8.4	5.1	233
Dipotassium tartrate.....	0.10	7.7	5.1	216
"    sulfate*.....	0.10	7.2	5.4	211
Disodium tartrate.....	0.10	7.3	4.9	180
"    sulfate*.....	0.10	6.8	5.2	103
"    oxalate.....	0.10	8.1	6.5	83
Sodium acetate.....	0.10	7.8	6.2	41
Potassium chloride.....	0.10	6.3		26
Sodium chloride.....	0.10	6.5	5.2	23
Potassium hydroxide*.....	0.015	11.8	7.8	229
Sodium hydroxide*.....	0.015	12.2	7.7	109

All of these salts were less effective in less concentrated solutions.

\* Made in the laboratory by mixing, in stoichiometric proportions, the same solution of KOH or of NaOH and the acid concerned.

extraction of this amylase from its activated alumina gel than the other salts here investigated. The influence of the anions appears to be much greater than that of the cations and no consistent trend of results with differences in the final hydrogen ion activity is observed. Thus, as shown in Table II, while the pH is approximately the same for the extracted solutions with tripotassium



citrate on the one hand and with disodium oxalate on the other, the extraction of the amylase is very different.

While the enzyme solutions used for the adsorption of the amylase in these two cases (Tables II and III) were made up to contain approximately the same activity per cc., their purity, or activity on the basis of total solids, was very different. The extract used

TABLE III

*Influence of Ions upon Extraction of Malt Amylase from Alumina Gel by Which It Had Been Adsorbed from Solution of Highly Purified Enzyme (Power Approximately 1500)*

Solution	Concentration	Original pH	Final pH	Enzyme activity Maltose per 0.1 cc.
	<i>mols per l.</i>			<i>mg.</i>
Dipotassium phosphate*.....	0.10	8.8	8.1	557
Tripotassium citrate*.....	0.10	7.4	7.1	479
Disodium phosphate*.....	0.10	8.8	8.1	463
Trisodium citrate*.....	0.10	7.6	7.2	395
Sodium potassium tartrate.....	0.10	8.4	5.7	91
Dipotassium tartrate.....	0.10	7.7	5.8	73
Disodium tartrate.....	0.10	7.3	5.8	65
Dipotassium sulfate*.....	0.10	7.2	5.7	55
Disodium sulfate*.....	0.10	6.8	5.9	16
Potassium chloride.....	0.10	6.3	5.1	7
Sodium chloride.....	0.10	6.3	5.0	5
Potassium chloride.....	0.01	5.3	4.9	77
Sodium chloride.....	0.01	5.4	4.9	76

With the exception of the chlorides as shown, all of the above salts were less effective in less concentrated solutions.

\* Made in the laboratory by mixing, in stoichiometric proportions, the same solution of KOH or of NaOH and the acid concerned.

for the adsorption in the experiment reported in Table II had an activity, on the basis of total solids, corresponding approximately to a power of 30 on the scale of Sherman, Kendall, and Clark (11), while the highly purified solutions used in the experiment reported in Table III had an activity of approximately 1500 on the same basis. In spite of this marked difference in the purity of the solutions of the enzyme used for the adsorption, the relative efficiency

of the different salts in the extraction of the amylase from the gel is in good agreement in the two cases.

Exceptions to this statement are found in the influence of the chlorides and in that of the hydroxides. The higher results obtained with the less concentrated solutions of the chlorides in the case of the more highly purified enzyme are not explained. At first glance, they might indicate that an impurity to which the enzyme is sensitive accompanied the salt and became more noticeable at the higher concentrations of the salt. As has already been pointed out, however, no indication of impurities was found by spectrographic analysis of the highly purified sodium chloride used or by any evidence that the salts influenced the activity of the enzyme.

When solutions of highly or even partially purified amylase preparations were treated with alumina gel, no measurable extraction of the amylase from the gel resulted by the use of sodium or potassium hydroxide but, as is shown in Table II, appreciable extraction resulted by their use when unpurified malt extracts were treated for adsorption. Apparently in these cases other anions present influenced the results.

#### DISCUSSION

The behavior in solution or in suspension of many complex inorganic salts and hydroxides such as those of chromium and of aluminum can be explained most satisfactorily by the application of the Werner (12, 13) theory and extensions of it which have been made by Bjerrum (14), Gustavson (15), Stiasny and coworkers (16), Thomas and his coworkers (4, 5), and others (17).

According to this theory thus extended the aluminum in alumina hydrate is present in the form of complex ionic micelles in which certain other groups are coordinately bound. In the simplest theoretical case, in aqueous suspension, each aluminum atom would have six coordinately bound aquo groups and the ionic micelle would carry a triple positive charge. The coordinately bound aquo groups are, however, readily replaced by other groups, especially hydroxo groups and the resulting basic cations readily combine through hydroxo, and under certain conditions, through oxo groups to form olated and oxolated compounds, so that large complexes tend to be formed. This has recently been discussed in detail by Thomas and Tai (5).

It has been shown that the anions of certain neutral salts will combine with basic aluminum complexes by displacing hydroxo groups. The degree of this displacement depends upon the penetration power of the anions added. By studies of their effectiveness in increasing the pH values of aluminum oxide hydrosols, Thomas and Tai found the penetration order of the anions studied by them to be: nitrate < chloride < acetate < sulfate < oxalate, tartrate. In the experiments reported here, taking the sodium salts at 0.10 M concentrations, the following order of effectiveness in the extraction of the amylase is obtained: chloride < acetate < oxalate < sulfate < tartrate < citrate < phosphate.

One of the theories (15) of chrome tanning of leather is that certain groups of the collagen molecule displace other coordinately bound groups in the complex chromium micelle forming a chromium compound in which certain groups of the protein molecules are inside the nucleus and the remainder outside. These coordinately bound groups may in turn be displaced by other groups of higher penetration capacity. This view offers the most reasonable explanation for the removal of chromium from chrome-tanned leather by treatment with sodium potassium tartrate solution.

A similar view applied to complex aluminum micelles would account for the findings reported in this paper. It is suggested that the enzyme is adsorbed by the alumina gel because certain groups of the molecule penetrate into the complex aluminum ionic micelle and displace other coordinately bound groups from it. Those anions which have a greater tendency to penetrate into the nucleus than the enzyme or its groups will displace it from the alumina gel while those with a lower capacity for penetrating into the nucleus will not.

The arrangement of the salts given here in the order of their increasing influence upon the extraction of malt amylase from the alumina gel by which it had been adsorbed is similar on the whole to that given by other workers for the increasing penetration action of the corresponding anions into complex ionic micelles of aluminum. The data given here are offered as showing an interesting extension of the recent work with complex ions, as an aid to others interested in the use of adsorption in the purification of malt amylase, and also as an indication that the adsorption of

malt amylase by alumina gel is governed by the same kind of chemical influences as those which control the union of more simple inorganic salts with complex ions like that of aluminum.

#### SUMMARY

Evidence is presented to show that the extraction of malt amylase from alumina gel by which it had been adsorbed is greatly influenced by the kind and concentration of anion present in the extracting solution and it is suggested that this may be correlated with an exchange of active enzyme from the aluminum ion complex for the anions of the electrolyte.

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## THE AVAILABILITY OF *d*-TRYPTOPHANE AND ITS ACETYL DERIVATIVE TO THE ANIMAL BODY

By VINCENT DU VIGNEAUD, ROBERT RIDGELY SEALOCK,  
AND CECIL VAN ETTEN

(From the Laboratory of Physiological Chemistry, University of Illinois,  
Urbana)

(Received for publication, August 15, 1932)

The study of the fate in the body of the optical isomers of the naturally occurring amino acids affords an interesting opportunity to bring to bear certain types of evidence on various problems in the field of metabolism of amino acids. By taking advantage of the essential character of certain amino acids for growth, such as cystine, histidine, lysine, and tryptophane, one has available an attractive method of studying the utilization of their isomers. The latter can be substituted for the naturally occurring form in the diet to test whether or not it is utilized for growth purposes.

Since we have been engaged in a study of the availability of *d*-cystine to the animal body from the standpoint just discussed, we wished to extend that type of study to other essential amino acids. The investigation of the utilization of *d*-tryptophane was therefore undertaken. The present report deals with the availability of *d*-tryptophane and its acetyl derivative.

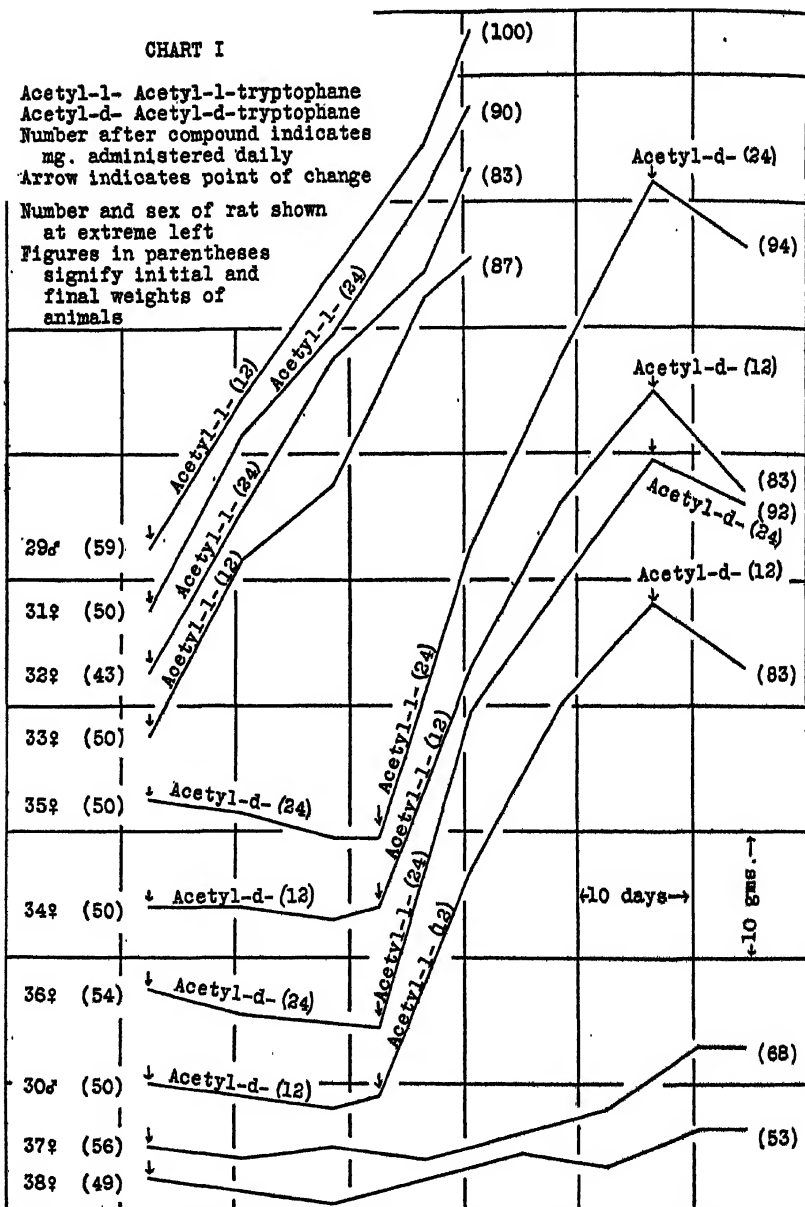
Since acetyl-*l*-tryptophane had already been shown to support growth by the work of Berg, Rose, and Marvel (1) and since the acetyl derivative of *d*-tryptophane was more readily available than the free amino acid in an optically pure state, it was decided to compare the growth response to the feeding of acetyl-*d*-tryptophane with that obtained with acetyl-*l*-tryptophane. If growth resulted from the feeding of the acetyl-*d*-tryptophane the question of the utilization of *d*-tryptophane would then, of course, be settled. On the other hand, negative results could not be considered conclusive, as we shall discuss later.

The results brought out in this comparative study as presented in Chart I show definitely that acetyl-*d*-tryptophane is not utilizable

### CHART I

Acetyl-l- Acetyl-l-tryptophane  
Acetyl-d- Acetyl-d-tryptophane  
Number after compound indicates  
mg. administered daily  
Arrow indicates point of change

Number and sex of rat shown  
at extreme left  
Figures in parentheses  
signify initial and  
final weights of  
animals



**CHART I. Rats 37 and 38 received no added tryptophane or derivative**

for growth purposes, in contrast to the acetyl derivative of the naturally occurring modification.

Since the lack of utilization of the dextro compound might have been due to a lack of absorption from the intestinal tract, both isomers were then administered subcutaneously. Again no growth was obtained with the acetyl-*d*-tryptophane, whereas excellent growth with the levo form was obtained.

Although these results seemed to indicate that *d*-tryptophane was not capable of being utilized by the animal body for growth, it was conceivable that the free *d*-tryptophane might be utilizable whereas its acetyl derivative might not. It is apparent that with the acetyl derivative hydrolysis in the body must occur before the question of utilization of the dextro or levo form can come into play. It is possible that this hydrolytic reaction, which is no doubt an enzymatic one, might be extremely specific with respect to spatial configuration. We have many instances where hydrolyzing enzymes are amazingly specific. Utilization, however, of the free *d*-tryptophane might occur by its being deaminized to an optically inactive keto acid which in turn could be asymmetrically synthesized by the body into the desired form of the amino acid. That the body can bring about such a reaction as the conversion of a keto acid back to an amino acid has been amply shown by various investigators, and particularly with respect to 3-indolepyruvic acid by Jackson (2) and by Berg, Rose, and Marvel (3). These investigators have shown that this compound can replace tryptophane in the diet. We should have, therefore, the question of the specificity of a hydrolytic reaction on the one hand and the specificity of a deamination reaction on the other. That deamination reactions of the animal body are not so strikingly specific is indicated by a number of examples in the literature. One might mention, for example, the observation made by Kapfhammer and Bischoff (4) that the amount of extra glucose obtained from both *l*- and *dl*-proline in the phlorhizinized dog was the same, and the finding of Kiech and Luck (5) that *dl*-alanine was almost completely deaminized within 12 hours.

The free *d*-tryptophane was therefore prepared and experiments were carried out comparing it with the levo form. The *d*-tryptophane which was obtained by hydrolysis of the acetyl-*d*-tryptophane by barium hydroxide had a rotation of  $[\alpha]_D^{30} = +31^\circ$ .



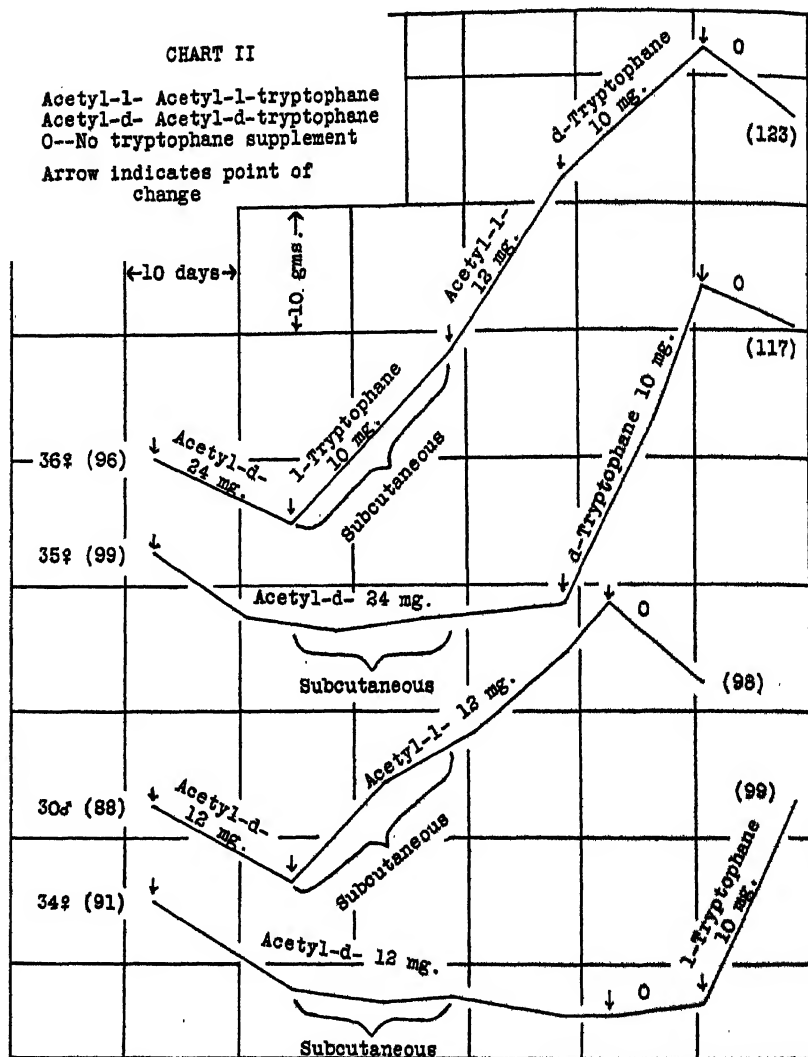


CHART II

The *l*-tryptophane isolated from an enzymatic hydrolysate of casein (6) gave under the same conditions a rotation of  $-32^{\circ}$ .

An immediate growth response to the feeding of the *d*-trypto-

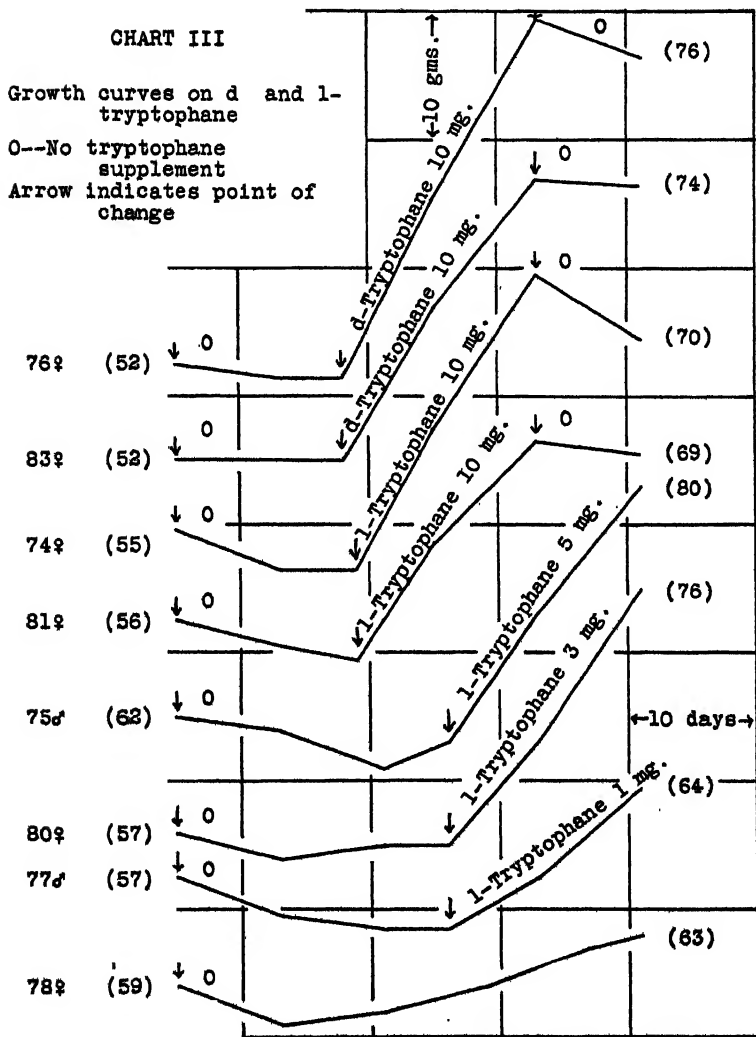


CHART III

phane was obtained as shown in Charts II and III. The growth of the animals on this isomer was just as good as that of the animals receiving *l*-tryptophane. The dextro enantiomorph was administered both to animals that had been on the deficient basal

diet and to animals that had been on a normal diet. In both types of experiments the dextro isomer replaced adequately the naturally occurring form. The average growth with *d*-tryptophane in all these experiments was 1.56 gm. per day, while that of the controls on *l*-tryptophane and in one instance acetyl-*l*-tryptophane during the corresponding periods was 1.42 gm.

In order to judge what response would result from a small amount of *l*-tryptophane a group of rats was given graded doses of *l*-tryptophane. It is evident from the growth curves of these animals in Chart III that the positive growth obtained with *d*-tryptophane cannot be attributed to a small amount of the levo isomer which might conceivably be present in the dextro sample.

These results on *d*-tryptophane are in essential agreement with the conclusions of Berg and Potgieter (7) who compared the growth-promoting powers of optically inactive tryptophane with *l*-tryptophane. They found that the *dl*-tryptophane was utilized to nearly as great an extent as the naturally occurring form.

The utilization of the injected acetyl-*l*-tryptophane would tend to show that the animal can hydrolyze the acetyl group independently of the intestinal tract although there is always present in such experiments the possibility of excretion into the intestine of the compound studied and reabsorption of the hydrolyzed product. The injection experiments demonstrate, however, that the lack of utilization of the acetyl-*d*-tryptophane cannot be due to a lack of absorption. It must be that the organism is incapable of hydrolyzing the acetyl group when it is linked to the unnatural isomer—a striking illustration of the specificity of hydrolytic reactions in the body with reference to spatial configuration. That this deamination reaction should be less specific than the hydrolytic reaction in question is suggestive that the deamination reaction is not an enzymatic one.

The formation of the hydroxy acid can be ruled out as an intermediary step in the conversion of the dextro to the levo isomer, if the work of Ichihara and Iwakura (8) is substantiated. These investigators claim that the indolelactic acid corresponding to *l*-tryptophane is utilizable for growth, whereas its optical isomer is not. If this be the case, the latter could not be considered as an intermediate compound in the conversion of the dextro form into the naturally occurring *l*-tryptophane.

The intermediary formation of the keto acid seems, on the other hand, to be a very likely explanation. The *d*-tryptophane would be oxidatively deaminized to the optically inactive keto acid from which *l*-tryptophane could be synthesized. We have no evidence that the body can directly racemize an optically active compound in order to use the other isomer from the resulting inactive mixture. Neither do we have any evidence of a direct conversion of one form to another.

These observations on *d*-tryptophane and acetyl-*d*-tryptophane have led us to plan an extension of this study to the physiological behavior and fate of the unnatural enantiomorphs of other amino acids, particularly of the essential amino acids in comparison with their corresponding acyl derivatives.

#### EXPERIMENTAL

The preparation of the acetyl derivatives of *l*- and *dl*-tryptophane and the resolution of the latter to give acetyl-*d*-tryptophane were carried out according to du Vigneaud and Sealock (9). The acetyl-*d*-tryptophane melted at 189–190° (corrected) and had a specific rotation of  $[\alpha]_D^{31} = -30.2^\circ$ .

The free *d*-tryptophane was obtained by the hydrolysis of acetyl-*d*-tryptophane. A 2 per cent solution of the latter in 10 per cent Ba(OH)<sub>2</sub> was refluxed for 2 hours. After the solution was cooled the barium was quantitatively removed as the sulfate. The filtrate was evaporated to dryness and the residue thoroughly extracted with absolute alcohol to remove any unchanged acetyl-tryptophane. The *d*-tryptophane was then recrystallized from 60 per cent alcohol. The rotation of a 0.5 per cent solution in water was  $[\alpha]_D^{30} = +31^\circ$ . Our *l*-tryptophane under the same conditions gave a rotation of  $-32^\circ$ .

The compounds were tested for their growth-promoting properties by being administered to young rats upon a tryptophane-deficient basal diet. The latter was composed of acid-hydrolyzed casein 14.7, cystine 0.3, dextrin 40, sucrose 15, lard 17, cod liver oil 5, salt mixture (Osborne and Mendel (10)) 4, and agar 2 per cent and was furnished *ad libitum*. A record was kept of food consumption.

The derivatives fed at 12 hour intervals were incorporated in the vitamin pills, which consisted of 150 mg. of yeast, 75 mg. of dextrin,

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and enough water to give a dough suitable for rolling into pills. Since the plan of the experiment was to feed the tryptophane and derivatives at a level equivalent to 10 and 20 mg. of tryptophane

TABLE I  
*Food Consumption and Body Weight Changes*

Rat No. and sex	Days	Average daily		Supplement*		Rat No. and sex	Days	Average daily		Supplement*	
		Change in weight	Food con- sump- tion					Change in weight	Food con- sump- tion		
		gm.	gm.		mg. per day			gm.	gm.		mg. per day
29 ♂	1- 28	+1.5	5.5	a-l	12	30 ♂	57-84	+0.79	4.7	a-l	12
31 ♀	1- 28	+1.4	4.9	a-l	24		85-92	-0.7	5.3		
32 ♀	1- 28	+1.4	4.8	a-l	24	37 ♀	1-72	+0.17	3.6		
33 ♀	1- 28	+1.4	4.6	a-l	12	38 ♀	1-72	0	2.7		
35 ♀	1- 20	-0.15	3.0	a-d	24	76 ♀	1-13	0	2.0		
	21- 44	+2.2	6.5	a-l	24		14-28	+1.9	3.6	d	10
	45- 80	-0.11	4.6	a-d	24		29-36	-0.4	2.2		
	81- 92	+2.1	6.6	d	10	83 ♀	1-13	0	2.3		
	93-100	-0.37	3.7				14-28	+1.5	4.0	d	10
34 ♀	1- 20	0	2.7	a-d	12		29-36	0	2.6		
	21- 44	+1.7	5.0	a-l	12	74 ♀	1-13	-0.23	2.2		
	45- 84	-0.2	3.4	a-d	12		14-28	+1.5	4.3	d	10
	85- 92	0	3.1				29-36	-0.6	2.7		
	93-100	+2.0	4.5	l	10	81 ♀	1-13	-0.23	2.2		
36 ♀	1- 20	-0.15	3.0	a-d	24		14-28	+1.6	3.7	l	10
	21- 44	+1.9	6.1	a-l	24		29-36	0	2.2		
	45- 56	-0.4	6.2	a-d	24	75 ♂	1-21	-0.2	2.6		
	57- 70	+0.71	5.0	l	10		22-36	+1.3	2.8	l	5
	71- 80	+1.8	6.7	a-l	12	80 ♀	1-21	0	2.3		
	81- 92	+0.83	5.8	d	10		22-36	+1.3	4.3	l	3
	93-100	-0.6	4.3			77 ♂	1-21	-0.2	2.2		
30 ♂	1- 20	0	3.4	a-d	12		22-36	+0.73	3.0	l	1
	21- 44	+1.6	5.7	a-l	12	78 ♀	1-36	+0.1	2.3		
	45- 56	-0.5	4.7	a-d	12						

\* l and d represent l- and d-tryptophane supplement and a-l and a-d the respective acetyl derivatives.

daily, some of these yeast pills were made up to contain either 5 mg. of tryptophane or 6 mg. of the acetyl derivative while other pills were made up to contain twice as much. Thus each experi-

mental animal would receive 300 mg. of yeast daily and either 10 or 20 mg. of tryptophane equivalent as the case might be. The control animals received 300 mg. of yeast daily but no extra tryptophane. Ten young rats from the same litter, varying in weight from 43 to 59 gm., were selected for the experiment. At the start of the work, of the four animals receiving acetyl-*d*-tryptophane, two received 12 mg. daily and the other two 24 mg. The four on the levo isomer were divided into two groups in the same way. The other two animals served as controls on the basal diet without added tryptophane.

The growth results are summarized in Chart I and the food consumption is shown in Table I. The animals receiving the acetyl-*d*-tryptophane showed no gain in weight and behaved similarly to the controls, whereas the animals on the acetyl-*l*-tryptophane grew rapidly. The animals receiving daily 12 mg. of the latter derivative showed almost as good growth as those receiving 24 mg., and therefore for the later part of this investigation the lower level for comparison was used. At the end of 20 days the animals receiving the dextro isomer were fed the levo isomer at the same level. Immediately these animals began to grow. Within 20 days they averaged about 40 gm. of gain in weight. At the end of this period they were put back again on the acetyl-*d*-tryptophane and immediately growth ceased; in fact, the animals began to lose weight.

At this point, of the four animals that had been receiving the dextro isomer two were given the compound by subcutaneous injection. Of the other two animals of this group one was given acetyl-*l*-tryptophane while the remaining animal received *l*-tryptophane, both compounds being administered subcutaneously. The daily dose was divided into four equal injections per day. A comparison of the results is given in Chart II, the first portion of the curves being a duplication for the sake of continuity of the latter part of the growth curves of these animals given in Chart I. After 14 days the subcutaneous injections were discontinued. Rat 36 which had been receiving *l*-tryptophane and Rat 30 which had been receiving acetyl-*l*-tryptophane were both given acetyl-*l*-tryptophane by pill. Rat 35 was continued on acetyl-*d*-tryptophane but the compound was given by mouth. The remaining animal in this group, which had been receiving acetyl-*d*-tryptophane, was placed on the basal diet.

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To one of the animals (Rat 36) that had been receiving acetyl-*l*-tryptophane, *d*-tryptophane was administered, while the other animal (Rat 30) was continued on the growth-promoting acetyl-*l*-tryptophane to serve as a control. Later both were given no added compound for a short period to demonstrate that the growth obtained was actually due to the effect of the compounds used. In order to test the effect of *d*-tryptophane on an animal that had been on the deficient diet, *d*-tryptophane was given to Rat 35 that had been receiving acetyl-*d*-tryptophane, and as a control to this Rat 34 which had also received acetyl-*d*-tryptophane was continued on the basal diet.

To another group of eight animals, all litter mates that had been for a period on the tryptophane-deficient diet, *l*-tryptophane and *d*-tryptophane were given, except to one animal which was continued on the basal diet. The results given in Chart III agreed uniformly with those given in Chart II, just as good growth being obtained with the dextro isomer as with the naturally occurring form. When the *d*-tryptophane and *l*-tryptophane were removed from the respective diets the animals stopped growing and began to lose weight.

### SUMMARY

It has been demonstrated that acetyl-*d*-tryptophane cannot be utilized for growth purposes whereas excellent growth was obtained with acetyl-*l*-tryptophane. Both feeding and injection of the derivatives led to the same results.

It has been shown that free *d*-tryptophane can be utilized for growth, the growth rate resulting from its administration being as great as that obtained with the naturally occurring *l*-tryptophane.

The possible significance of these findings with respect to the specificity of spatial configuration from the standpoint of hydrolytic reactions in contrast to deamination reactions in the body has been discussed.

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## A COMPARISON OF THE GROWTH-PROMOTING PROPERTIES OF *d*- AND *l*-CYSTINE

BY VINCENT DU VIGNEAUD, RALPH DORFMANN, AND  
HUBERT S. LORING

(From the Laboratory of Physiological Chemistry, University of Illinois,  
Urbana)

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The isolation of the pure meso- and *dl*-cystine and the resolution of the latter into its dextro and levo components (1, 2) have made available for biochemical study all the stereoisomeric forms of this interesting amino acid. Such a study has within it the possibility of yielding information on the intermediary metabolism of cystine and contributing further data on the specificity of biological reactions with respect to spatial configuration.

The present report will be concerned with the question of whether or not the dextro isomer can be utilized for growth purposes in lieu of the naturally occurring *l*-cystine. We hope to be able to present at a later time a similar investigation of mesocystine and further a study of the catabolic fate of both of these stereoisomers.

The *d*-cystine used in these studies was obtained from the resolution of inactive cystine by means of the brucine salt of the acetyl derivative (1) and from the resolution of pure *dl*-cystine by means of the strychnine salt of the formyl derivative (2).

The presence of mesocystine in the inactive mixture of cystine obtained by the racemization of *l*-cystine with acid obviously hinders somewhat the resolution of the racemic component, and it is therefore preferable to isolate first the pure racemic component from the inactive cystine mixture. This isolation can be accomplished very readily by fractional crystallization of the inactive cystine hydrochlorides. The resolution of the *dl*-cystine can be effected by means of the strychnine salt of the formyl compound. The preparation of the latter according to the method of Professor Hans T. Clarke is simpler to carry out than the preparation of

acetylcystine. The formylcystine has a further advantage in that it is even more readily hydrolyzed than the acetyl derivative and gives, therefore, less opportunity for racemization during hydrolysis.

There has recently appeared an investigation by Lawrie (3) in which the growth obtained with inactive cystine prepared by acid racemization is compared with that obtained with the levo modification. Lawrie came to the conclusion that the value of inactive cystine for tissue-building purposes lies between one-third and two-thirds that of *l*-cystine.

#### EXPERIMENTAL

##### *Isolation of Pure dl-Cystine*

The inactive cystine was prepared by the racemization of *l*-cystine by boiling with concentrated HCl according to the method of Hoffman and Gortner (4). The length of time, however, of refluxing was lengthened to 5 days. The solution of the resulting inactive cystine was then directly concentrated *in vacuo* and three successive fractions of fairly pure crystalline *dl*-cystine hydrochloride were removed. The mesocystine hydrochloride is much more soluble and the majority remains in the mother liquor. The *dl*-cystine hydrochloride fractions were purified by further recrystallizations by dissolving them in water with enough HCl added to prevent free cystine from separating and then adding concentrated HCl so that the resulting acid concentration was approximately 20 per cent and the cystine hydrochloride about 8 per cent. Upon standing and cooling in an ice bath the *dl*-cystine hydrochloride crystallized. In a series with about 200 gm. of cystine the three fractions mentioned above contained 50, 40, and 40 gm., respectively. The first fraction upon one recrystallization yielded 30 gm. of pure *dl*-cystine hydrochloride. The second fraction gave 15 gm. and the third fraction 5 gm. after two and three recrystallizations respectively. Another 10 gm. were obtained by working up the mother liquors from these recrystallizations; the total yield was 60 gm. of pure *dl*-cystine hydrochloride.

The *dl*-cystine hydrochloride was converted to the free *dl*-cystine by dissolving it in a large volume of water and neutralizing the solution with  $\text{NH}_4\text{OH}$ .

# Analysis

3.425 mg. substance: 0.368 cc. N at 35° and 751 mm.

4.856 " " : 9.39 mg. BaSO<sub>4</sub>

C<sub>6</sub>H<sub>12</sub>O<sub>4</sub>N<sub>2</sub>S<sub>2</sub>. Calculated. N 11.67, S 26.66

Found. " 11.75, " 26.55

If one's interest is mainly in obtaining only the racemic form and not the meso form, further amounts of *dl*-cystine are obtainable by reducing the mesocystine-containing mother liquors to *dl*-cystine by tin in the usual fashion and then after the removal of the tin as the sulfide reoxidizing the material by bubbling air through the solution. We have found that upon oxidation the *dl*-cystine yields a mixture of the *dl*- and mesocystine. The *dl*-cystine can be separated as described above, the mixture behaving similarly to the original inactive mixture obtained by acid racemization.

Another possible method of obtaining more of the racemic form from the original batch of cystine is by further refluxing the mesocystine-containing mother liquors with concentrated HCl, converting some of the meso component to the racemic form.

The fractionation of these hydrochlorides is greatly aided by following the various steps by microscopic examination of the crystals. The *dl* hydrochloride crystallizes in characteristic diamond-shaped crystals, often with two opposite corners cut off, making actually a six-sided crystal yet retaining the distinct diamond-like form. The meso form on the other hand crystallizes in short blunt prisms.

## Preparation of *dl*-Formylcystine

40 gm. of pure *dl*-cystine were placed in a 1 liter three-necked round bottom flask fitted with a mechanical stirrer, condenser, and separatory funnel, and 600 cc. of 85 to 90 per cent formic acid added. The flask was warmed to 50–60° and after solution of the cystine 200 cc. of acetic anhydride were added by means of the separatory funnel, at such a rate that the liberated heat maintained a temperature of approximately 60°. Usually approximately half an hour is required for the addition of the acetic anhydride. The solution was then allowed to cool slightly and 100 cc. of water were added to decompose any remaining acetic anhydride. The solution was then cooled further in an ice bath and approximately

28 gm. of *dl*-diformylcystine separated. This was filtered and the mother liquor concentrated *in vacuo*, preferably after further dilution with water, to a small volume from which a second crop of about 8 gm. of crystals precipitated. This was then combined with the first crop and recrystallized from the minimum amount of water, 27 gm. of the pure compound being obtained. The product melted at 194–196°.

*Analysis*

3.053 mg. substance: 0.258 cc. N at 25.5° and 746 mm.

$C_8H_{12}O_6N_2S_2$ . Calculated, N 9.46; found, N 9.48

*Resolution of dl-Diformylcystine*

33 gm. of *dl*-diformylcystine were converted to the strychnine salt by the alternate addition of the formyl derivative and strychnine to 500 cc. of boiling water, a total of 80 gm. of strychnine being used. The hot solution was then filtered from any undissolved strychnine and allowed to cool in an ice bath. A large yield of the strychnine salt was obtained which was then recrystallized three times from water, a little more than the minimum amount of hot water being used each time to dissolve the material. A yield of 31 gm. of the pure *d* salt was obtained. The product melted at 182–183° (corrected) and had a specific rotation of  $[\alpha]_D^{20} = +22^\circ$ . By further working up of the mother liquors in the usual fashion 4 more gm. of the pure *d* salt were obtained. It might be pointed out that unlike the behavior of the brucine salt of the acetyl derivative recrystallization of fractions predominantly rich in the *l* salt led to the separation of the *l* salt. The *d* salt contains 3 molecules of water of crystallization.

*Analysis*

3.139 mg. substance: 0.249 cc. N at 27° and 744 mm.

$C_{10}H_{16}O_{10}N_2S_2$ . Calculated, N 8.72; found, N 8.83

*Hydrolysis of Diformyl-d-Cystine*

30 gm. of the strychnine salt of diformyl-*d*-cystine were dissolved in 1 liter of warm water, and the solution was cooled in ice. To the cooled solution 6 cc. of concentrated  $NH_4OH$  were added slowly with stirring. The precipitated strychnine was filtered and washed with cold water containing a few drops of  $NH_4OH$ .

The filtrate and washings were made slightly acid to Congo red with HCl and concentrated *in vacuo* to a small volume. The residue of diformyl-*d*-cystine was taken up in 50 cc. of hot 1 *N* HCl and the solution refluxed for 15 minutes in an oil bath at 130–140°. The acid solution was then concentrated *in vacuo* almost to dryness. The *d*-cystine hydrochloride was dissolved in a small volume of water and the solution neutralized with NH<sub>4</sub>OH. The *d*-cystine which precipitated could then be recrystallized by dissolving it in dilute HCl and precipitating it by neutralization with NH<sub>4</sub>OH. The yield of purified product was 7.2 gm. The *d*-cystine gave a specific rotation of  $[\alpha]_D^{25} = +208^\circ$ .

### *Growth Experiments*

The *d*-cystine was tested for its growth-promoting properties by being administered to young rats upon a cystine-deficient diet. Much effort was expended searching for a cystine-deficient diet on which without added cystine the animals would consistently grow at a slow enough rate so that upon the addition of *l*-cystine to the diet a clean cut increased rate of growth would result. Preliminary experiments were run in which casein, whole milk powder, or cystine-free hydrolyzed casein and yeast were used. These showed a rate of growth on the basal diets greater than was desired for our purposes. This was found to be the case also with the navy bean diet of Johns and Finks (5).

Better results were obtained by using a yeast concentrate in place of the yeast. This concentrate was one prepared by The Fleischmann Laboratories and kindly made available to the authors by Professor W. C. Rose. This concentrate was used in conjunction with hydrolyzed casein which had been treated with silver according to the method of Kossel and Kutscher (6). We found, as would be expected from the work of Vickery and Leavenworth (7), that this procedure yielded a cystine-free product. It was necessary, of course, in the feeding work to supplement the material with histidine and tryptophane.

Preliminary experiments were first carried out to find out the level at which the yeast concentrate would cure polyneuritis and give normal growth. A group of animals was allowed to develop symptoms of polyneuritis and was then given the yeast concentrate. It was found that about 100 mg. of yeast concentrate were

necessary daily for normal growth over a long period of time, although a much smaller dose sufficed to clear up the polyneuritic symptoms. The concentrate contained 28 per cent moisture in contrast to the yeast used in the experiments mentioned above which contained 10 per cent.

TABLE I  
*Food Consumption and Body Weight Changes*

Rat No. and sex	Days	Average daily		Diet*	Rat No. and sex	Days	Average daily		Diet*
		Change in weight	Food con- sump- tion				Change in weight	Food con- sump- tion	
		gm.	gm.				gm.	gm.	
40 ♀	1- 60	+1.17	4.4	III + <i>l</i>	22 ♀	1- 64	+0.94	4.6	I + <i>l</i>
	60- 88	-0.14	4.1	III		64-102	-0.08	3.6	I + <i>d</i>
	88- 96	+1.75	5.0	III + <i>l</i>		102-128	+0.96	4.9	I + <i>l</i>
39 ♂	1- 60	+1.20	5.1	III + <i>l</i>	23 ♀	1- 24	+0.33	4.5	I
	60- 88	-0.21	4.3	III + <i>d</i>		24- 62	+1.11	4.7	I + <i>l</i>
	88- 96	+2.25	6.5	III + <i>l</i>		62-108	-0.09	4.3	I + <i>d</i>
21 ♀	1- 64	+1.12	4.7	I + <i>l</i>	24 ♀	1- 64	+0.41	3.0	I
	64-102	-0.08	4.5	I		64-102	+0.45	4.0	I + <i>d</i>
	102-128	+1.04	5.4	I + <i>l</i>		102-128	+0.89	4.5	I + <i>l</i>
56 ♂	1- 64	+1.67	8.7	II + <i>l</i>	53 ♂	1- 64	+0.17	6.0	II
	64- 72	-3.50	6.6	II + <i>d</i>		64- 72	+2.50	9.1	II + <i>l</i>
50 ♀	1- 48	+1.48	7.5	II + <i>l</i>	55 ♀	1- 28	+0.07	5.8	II
	48- 72	-0.42	5.8	II + <i>d</i>		28- 72	+0.23	5.5	II + <i>d</i>
49 ♂	1- 28	0	4.8	II	52 ♂	1- 28	+1.00	6.5	II + <i>l</i>
	28- 64	+0.45	6.0	II + <i>d</i>		28- 64	0	6.0	II + <i>d</i>
	64- 72	+3.25	11.3	II + <i>l</i>		64- 72	+3.38	10.5	II + <i>l</i>
51 ♀	1- 72	+0.06	5.6	II	72 ♀	1- 20	-0.15	2.7	II
70 ♀	1- 20	-0.35	3.2	II		20- 40	+0.25	3.8	II + <i>d</i>
	20- 40	+1.10	4.0	II + <i>l</i>		20- 40	-0.40	2.9	II
73 ♂	1- 20	-0.30	3.4	II	71 ♂	1- 20	-0.40	2.9	II
	20- 40	+1.30	5.4	II + <i>l</i>		20- 40	-0.05	3.3	II + <i>d</i>

\* *l* and *d* represent *l*-cystine and *d*-cystine supplements respectively.

Encouraging enough results were obtained with the above combination to warrant its use in the comparison of the dextro and levo isomers. Diet I, used in these experiments, had the following composition: cystine-deficient hydrolysate of casein 14.0, histidine 0.4, tryptophane 0.2, dextrin 40.0, sucrose 15.0, lard 19.0, cod

liver oil 5.0, salt mixture (Osborne and Mendel (8)) 4.0, and agar 2 per cent. It was fed *ad libitum* and a record kept of the food consumption. The yeast concentrate and cystine when fed were administered in the form of pills, the daily amount being divided into three equal portions.

One litter of four rats was used in the experiment on this diet. Two of the animals were placed on the basal diet with no added cystine and two were given 20 mg. of *l*-cystine per day. After the latter animals had been on that diet for 2 months the *l*-cystine feeding was stopped and one of the animals (Rat 22) was given *d*-cystine while the other (Rat 21) was given no added cystine. Both animals stopped growing and began to lose weight. Later they both showed a slight recovery of the loss but even after almost 6 weeks their weight was still below that which it had been at the time the *l*-cystine was taken away. It is quite apparent from Chart I that no better growth was obtained with the *d*-cystine than without cystine. When *l*-cystine was given to both animals towards the end of the experiment both rats responded similarly with marked increases in growth. Of the two animals that had been placed originally on the basal diet, Rat 24 was given *d*-cystine and the other (Rat 23) was given *l*-cystine. As shown in Chart I the addition of *d*-cystine did not change the rate of growth whereas *l*-cystine caused an increase. After they had been on this diet for about 2 months the one that had been receiving *l*-cystine was given *d*-cystine and the one on *d*-cystine was given *l*-cystine. The former lost weight for a time and then maintained itself at this lower level for a period of 6 weeks. When *l*-cystine was administered at this point the animal began to grow again. When the remaining animal that had been on *d*-cystine was given the levo enantiomorph a marked stimulation in growth resulted.

The growth, however, on the basal diet was still a little more than desired and it was decided to go back to the vitamin supplement and procedure used by Osborne and Mendel (9) in their classical study on the essential nature of cystine. In this investigation "protein-free milk" was utilized for the source of the water-soluble vitamins, and as a source of protein casein was used at a level sufficient to supply enough nitrogen for normal growth when cystine was added. Through a suggestion from Professor Mendel for which we wish to express our appreciation, we have tried in



CHART I.

GROWTH CURVES ON DIET I.  
Litter A-Rats 21, 22, 23 and 24.

GROWTH CURVES ON DIET III.  
Litter B-Rats 39 and 40.

Cystine supplement 20mg.daily.  
Arrows indicate change.

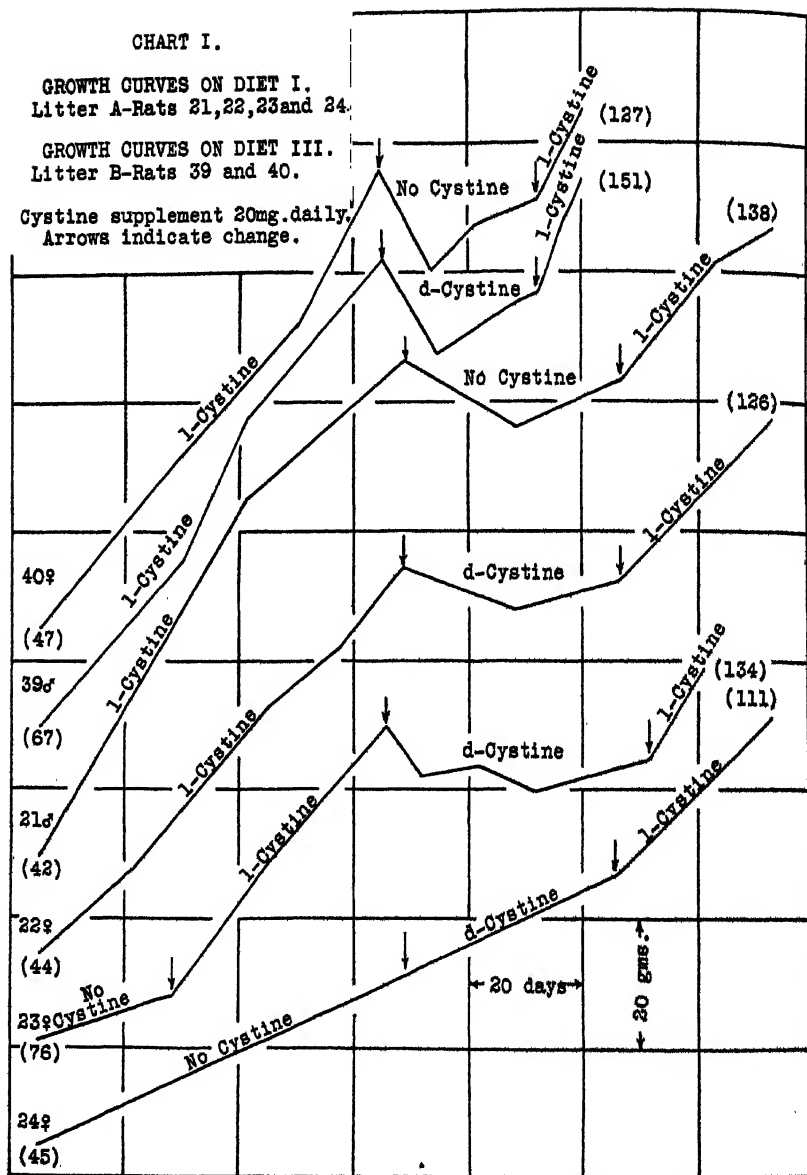


CHART I. The numbers in parentheses denote the initial and final weights of the rats.

place of "protein-free milk" the milk vitamin concentrate of Supplee<sup>1</sup> (10) with excellent results. The animals on the basal diet showed little growth and upon the administration of *l*-cystine a clean cut increased rate of growth resulted; this affords an excellent diet for cystine replacement studies. Our Diet II which contained the milk vitamin concentrate was made up as follows: casein 8.0, dextrin 31.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Osborne and Mendel (8)) 4.0, agar 2.0, and milk vitamin concentrate 16.0. The amount of concentrate used was based on the work of Supplee, Kahlenberg, and Flanigan (10). The diet was fed *ad libitum* and a record kept of food consumption. When cystine was administered, it was given in the form of pills with enough dextrin and water to form a thick paste. The daily amount was divided into three portions.

For this series of experiments with Diet II, two rats of a group of four litter mates were placed on the basal diet without added cystine while the other two were given 20 mg. of *l*-cystine per day. 4 weeks later one of the latter pair (Rat 52) was given 20 mg. of *d*-cystine in place of the levo isomer while the other (Rat 56) was allowed to continue on the levo form. As shown in Chart II, Rat 52 stopped growing and after 5 weeks still showed the same weight whereas the control Rat 56 on *l*-cystine continued to grow, gaining 67 gm. in this same interval of time. At this point Rat 52 was given *l*-cystine. A marked stimulation in growth resulted. The animal gained 26 gm. in 1 week, whereas it had shown no gain during 5 weeks on the dextro isomer. At the same time Rat 56 which had been gaining nicely during the 5 week period on *l*-cystine was given a like amount of *d*-cystine. The effect was striking. Within 1 week the animal lost 28 gm. Of the pair placed originally on the basal diet one was given *d*-cystine after 4 weeks while the other was continued as a control without the addition of cystine. The rat receiving *d*-cystine showed no greater growth rate than the control animal.

A group of three rats from another litter was also placed on the basal diet, one of them receiving in addition 20 mg. of *l*-cystine daily. This latter animal was allowed to continue on *l*-cystine

<sup>1</sup> The authors wish to thank Dr. Supplee of the Research Laboratories of The Dry Milk Company for a liberal supply of the milk vitamin concentrate which was used in this investigation.

CHART II.

GROWTH CURVES ON DIET II.  
Litter C-Rats 49, 50 and 53.  
Litter D-Rats 51, 52, 55 and 56.

Cystine Supplement 20mg. daily.  
Arrows indicate change.

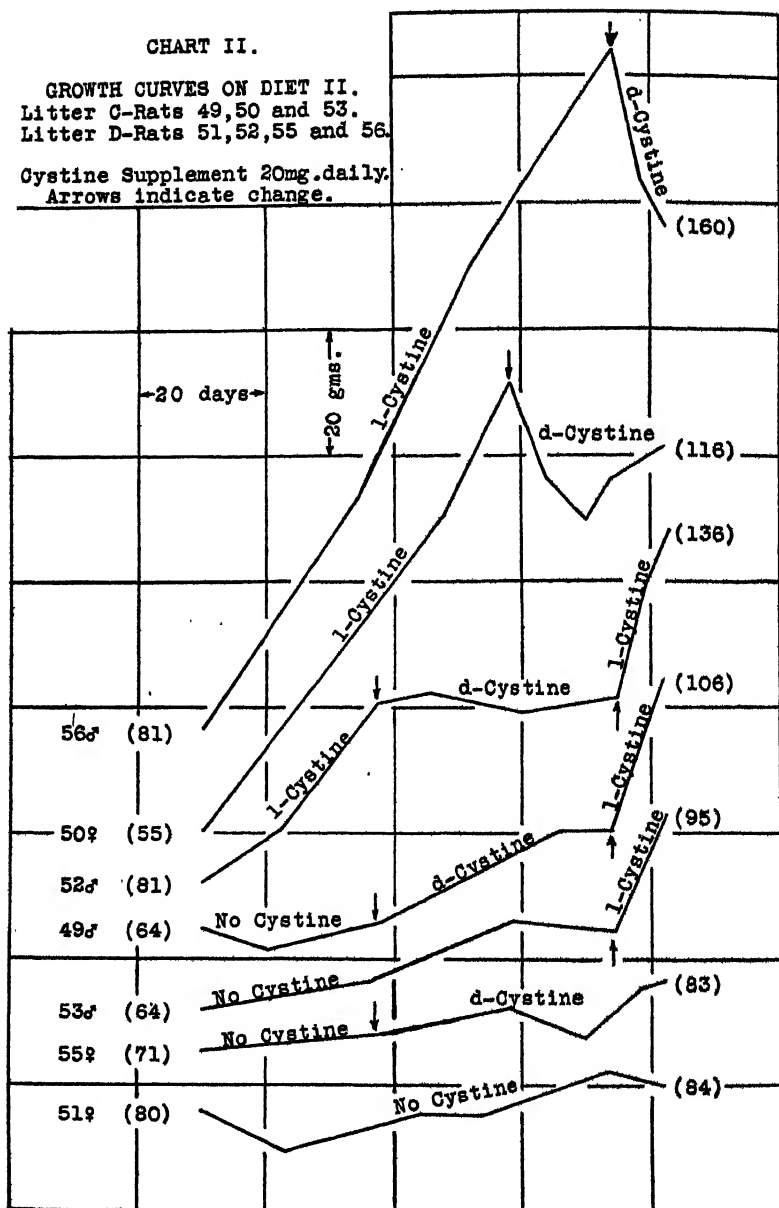


CHART II. The numbers in parentheses denote the initial and final

for about 7 weeks, at which time it was replaced with *d*-cystine. An immediate decline in weight set in with slow recovery similar to what we have seen above. The other two animals of this litter were kept on the basal diet for 4 weeks, and then one was given 20 mg. of *d*-cystine daily, the other being continued on the basal

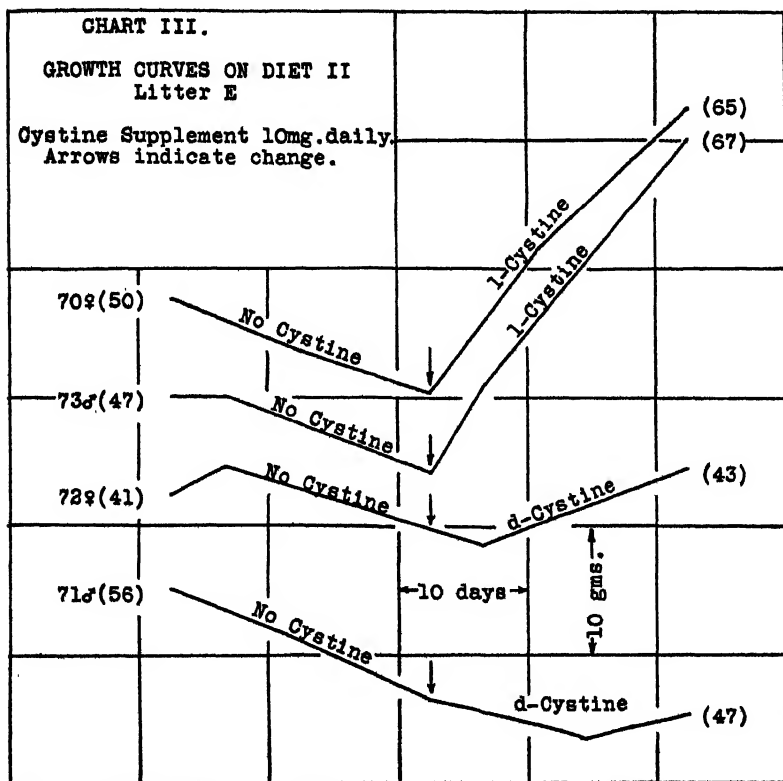


CHART III. The numbers in parentheses denote the initial and final weights of the rats.

diet. No change in growth rate seemed to occur. After the former had been receiving *d*-cystine for a little over 5 weeks *l*-cystine was substituted for the dextro isomer and at the same time the control was also given *l*-cystine. Both animals responded similarly with marked stimulation in growth, the animal that had

previously been on *d*-cystine gaining 26 gm. in 8 days while the control gained 20 gm.

One pair of females and one pair of males of another litter were placed on the basal diet for 20 days, at which time one of the males and one of the females was each given 10 mg. of *l*-cystine daily and the other pair 10 mg. of *d*-cystine. As shown in Chart III the animals on the levo isomer gained 25 gm. in one case and 23 in the other during the following 20 days, whereas one of the animals on the dextro enantiomorph actually weighed 1 gm. less than at the start of this period and the other gained only 4 gm.

The milk vitamin concentrate was also used in connection with the silver-treated casein hydrolysate. This diet, Diet III, was identical with Diet I except for the vitamin B and vitamin G supplementation. Supplee *et al.* (10) have shown that 200 mg. of this concentrate will suffice for normal growth if supplemented with an additional amount of the antineuritic substance. This amount of concentrate was therefore tried, supplemented with 0.5 mg. of an antineuritic concentrate of Seidell which Dr. Seidell very generously gave us. It might be well to emphasize that with Diets I and III the vitamin B and vitamin G supplement was given separately from the main diet in the form of pills and that with Diet II it was mixed with the main diet. In so far as the question of the replaceability of *l*-cystine by *d*-cystine was concerned, no difference in the results was shown. The pair of animals used on this diet was kept on the basal diet plus 20 mg. of *l*-cystine daily for 2 months, and then one of them was given 20 mg. of *d*-cystine in place of the *l*-cystine for a period of 1 month while the other during the latter period received no cystine supplement. Both animals responded similarly, the animal receiving the dextro isomer faring no better than the control. They were then both given *l*-cystine and both responded with marked growth.

#### DISCUSSION

All of our experiments designed to test whether *d*-cystine could be utilized by the body for growth purposes, with the three types of diets described, consistently led to the conclusion that the dextro isomer cannot be utilized. The results pointed to the same conclusion whether the *d*-cystine was administered to rats that had been growing normally on *l*-cystine or whether it was given

to animals that had been on a cystine-deficient diet. In the former case the normal growth rate was strikingly arrested; in fact, the animals actually lost weight for a time and then started to grow again at a decidedly decreased rate. This behavior was identical to that of control animals from whose diet the *l*-cystine had been likewise removed but to which no extra cystine was given. Addition of *d*-cystine to the diet of animals growing slowly on the cystine-deficient diet did not seem to increase significantly the rate of growth above the rate at which the animals had been growing or above that of control animals which had been continued on the cystine-deficient diet. This is quite in contrast to what we have found from the study of the availability of *d*-tryptophane from the growth standpoint. As good growth was obtained by the administration of *d*-tryptophane to animals on a tryptophane-deficient diet as with the naturally occurring levo enantiomorph.

#### SUMMARY

The isolation of pure *dl*-cystine has been described.

A method of resolving *dl*-cystine by means of the strychnine salt of the formyl derivative has been presented.

It has been demonstrated that *d*-cystine cannot be utilized for growth purposes in lieu of the naturally occurring levo enantiomorph.

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## THE PREPARATION OF PURIFIED PROGESTIN

By WILLARD M. ALLEN

*(From the Department of Anatomy, The University of Rochester School of Medicine and Dentistry, Rochester, New York)*

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Since our last publication (Allen, 1930,*a*) giving a method for the preparation of progestin, sufficient added information has been accumulated to warrant the publication of the procedure we now follow. The main difficulties encountered in the purification of progestin remain much the same as at that time and seem likely to remain since they depend fundamentally on the behavior of this hormone with respect to various solvents and reagents.

Methods of extraction differing from the boiling neutral alcohol method previously published have been tried but only one need be given—and that only because it may eventually offer a better method of preparation than the one to be given subsequently in this paper. In this method, benzene was used as the extracting agent because of its success in the hands of Laqueur (1927) in the preparation of menformone and because of the fact that it separates the proteins from the fats readily and does away with the large amount of breakdown products formed by boiling alcohol. The one objection to benzene is, of course, that it penetrates tissue poorly because of its relatively slight solubility in water. To counteract this the fresh tissue was finely minced and then ground with sea sand. Extractions with both hot and cold benzene were tried (Table I), and surprisingly enough two extractions with benzene in some cases gave a complete extraction of the hormone, leaving none that could be extracted from the residue by boiling alcohol. However, in other extracts there was a considerable amount of hormone remaining in the residue after benzene extraction. The use of benzene was discontinued largely because of the irregularity of results even though it might probably with more adequate study be found to be as good as or even better than the boiling alcohol method.



The method previously described has been subjected to detailed study, each step being more thoroughly investigated. Formerly we extracted the tissue with boiling alcohol in Bloor extractors five times, each extraction being continued for 1 hour. We also used fresh alcohol for the extracting fluid. It has since been found that three extractions are sufficient to extract all of the progesterin, provided that the tissue in the bags is broken up by kneading between each extraction and the alcohol is kept boiling vigorously, and also that the alcohol used for preserving the tissue

TABLE I  
*Yields Obtained by Extracting Raw Material with Benzene*

Ex- tract No.	Fresh tissue	Type of extraction	Yield		Yield from boiling alcohol extraction of benzene-insoluble
			<i>rabbit units per kg.</i>		<i>rabbit units per kg.</i>
77	360	Extracted with 2 volumes hot benzene 2 times, 1 hr. each	++++ >34.7	++ <69.4	+ <14
79	250	Extracted with 2 volumes cold benzene 2 times, 12 hrs. each	++++ >40		++ <14
80	608	Extracted with cold ben- zene 12 hrs., then with hot benzene 1 hr.		++ <20.5	++++ >7.5 Probably 15
81	350	Extracted with 2 vols. hot benzene 1 hr. each		++ <47.4	0 <6

The + signs over the figures denoting yields represent the degree of proliferation produced by injection of a dose corresponding to that yield. For example, 4+ above 34.7 indicates that a dose giving 4+ proliferation was injected on the basis of 34.7 rabbit units per kilo. The 0 above a figure means no proliferation was produced by that dosage.

may be used for the extraction. The next important simplification has been developed in the stage in which the phospholipids are removed by acetone. Formerly these were precipitated by acetone, redissolved in ether, and reprecipitated by acetone six times to assure that none of the progesterin was lost by adsorption to this bulky precipitate. It has been found, however, that two precipitations of the phospholipids are sufficient provided the solution containing the precipitate is thoroughly agitated and allowed to stand for several hours before filtering. That these

modifications cause no loss in yield is shown in Table II. For example, the fourth and fifth alcoholic extractions of the tissue in Bloor extractors contained so little hormone that injection of an equivalent of 1232 gm. of fresh tissue produced no proliferation. The phospholipids, also, after two precipitations were found to be inactive when an equivalent of 628 gm. of fresh tissue was injected, whereas the acetone-soluble fraction was active in an equivalent dose of 25 gm. of fresh tissue.

The purification of the crude oil was formerly accomplished by repeated freezing from absolute methyl alcohol and then by partitioning the methyl alcohol-soluble substances between petroleum

TABLE II  
*Inactivity of Discarded Fractions in Stages (d) and (h)*

Extract No.	Tissue extracted	Amount of hormone recovered by 4th and 5th extractions with boiling alcohol of residue (d)			Amount of hormone remaining in the phospholipid fraction after 2 precipitations with acetone (h)		
		Equivalent of tissue injected	Resultant proliferation	Loss*	Equivalent of tissue injected	Resultant proliferation	Loss*
	gm.			rabbit units per kg.			rabbit units per kg.
74	1950				234	0	0
84	2157	1232	0	0	513	0	0
85	1885	943	0	0	628	0	0

The italic letters, (d) and (h), refer to the steps in the diagrammatic outline.

\* The potent fraction at each of these steps was active with a dose equivalent to 25 gm. of fresh tissue.

ether and 70 per cent alcohol, a definite amount of 70 per cent methyl alcohol solution being extracted five times with one-third its volume of fresh petroleum ether. This sufficed to remove practically all of the cholesterol from the 70 per cent alcohol fraction and thereby effected a marked purification of the hormone, but at the same time some of the hormone was always found in the petroleum ether fraction. The results from four different extracts (Table III) which were carefully assayed show that there was a 75 per cent recovery of the hormone in the alcohol fraction. From this limited evidence and the use of a distribution formula

such as that used by Ralls, Jordan, and Doisy (1926) in studying cholesterol distribution between these solvents it was found that progesterin under the conditions of this experiment was approximately 5 times as soluble in 70 per cent methyl alcohol as in petroleum ether. From this finding, and from the known fact that cholesterol and neutral fat are relatively insoluble in dilute alcohol, it seemed possible to eliminate this process of freezing from absolute alcohol with subsequent distribution between two solvents as a method for the separation of fat and cholesterol, and to substitute for it freezing of a 70 per cent methyl alcohol solution. This procedure has been found to be very efficacious in that in one

TABLE III

*Distribution of Progesterin between 70 Per Cent Methyl Alcohol and Petroleum Ether after Extracting the Alcoholic Solution Five Times with One-Third Its Volume of Petroleum Ether. Alcoholic Fraction Cholesterol-Free*

Preparation No.	Petroleum ether		70 per cent methyl alcohol		Recovery in 70 per cent alcohol
	Rabbit units	Weight of rabbit unit	Rabbit units	Weight of rabbit unit	
		gm.		gm.	per cent
46	10	0.450	25	0.040	71.4
52	5	1.120	15	0.026	75.0
59	5	0.500	10	0.0058	66.6
72	5	0.033	25	0.0026	83.3
Average.....					74.0

step it removes the cholesterol completely, large amounts of neutral fat, and practically none of the hormone. The technical difficulties encountered in separating fats from dilute alcohol were overcome by filtration after cooling at  $-4^{\circ}$  for 12 hours.

This procedure is carried out as follows:<sup>1</sup> 140 cc. of boiling absolute methyl alcohol are added to the acetone-ether-soluble fraction (i) and the flask agitated thoroughly so that a fine emulsion of the fat is obtained. 60 cc. of water are then added and

<sup>1</sup> These directions are based on the extraction of 1500 gm. of tissue and they, together with the outline and the specific directions previously published (Allen, 1930, *a*), may be fitted easily into the complete method of preparation.

Hand-dissected tissue is preserved in 2 volumes of 95 per cent alcohol. It may be kept in this way for at least a year. All subsequent volumes are on the basis of 1500 gm. of tissue. When ready to extract, filter through gauze bags in Bloor extractors.

#### Filtrate (a)

#### Residue (b)

Divide in 3 equal portions and extract each 3 times in Bloor extractor with filtrate (a) for the extracting fluid

#### Hot alcohol-soluble (c)

#### Residue (d)

Distil to a thick sludge (water pump vacuum) and extract with ethyl ether; 500 cc., 300 cc., 300 cc.

Protein  
(discard)

#### Ether-soluble (f)

#### Ether-insoluble (g)

All forms of fatty substances. Distil to 100 to 200 cc., then add 800 cc. of acetone and 10 cc. of saturated alcoholic solution  $MgCl_2$ . Dissolve precipitate in ether and reprecipitate once

Protein split-products  
(discard)

#### Acetone-soluble (i)

#### Acetone-insoluble (h)

Distil to a thick oil *in vacuo* for so called crude oil. Dissolve in alcohol. Yield 30 gm., 60 rabbit units

Phospholipids  
(discard)

Or distil to a thick oil and extract with 200 cc. of hot 70 per cent methyl alcohol, cool at  $-4^\circ$  for 12 hrs., and filter. Reextract precipitate with hot alcohol, cool, and filter 3 more times

#### Cold methyl alcohol-soluble (j)

#### Cold methyl alcohol-insoluble (k)

Distil to about 5 cc. *in vacuo*, add 100 cc. absolute ethyl alcohol, and distil to dryness. Yield 2 gm. 1 rabbit unit = 30 to 40 mg. Dissolve in 50 cc. alcohol as a stock solution

Neutral fat and cholesterol  
(discard)

Or distil to about 20 cc. and extract with ethyl ether; 100 cc., 50 cc., 50 cc.

#### Ether fraction (l)

#### Aqueous fraction (m)

Wash with saturated aqueous  $NaHCO_3$ , then 0.2 N HCl, and then with  $H_2O$

(Discard)

#### (Ether fraction (o))

#### Washings (p)

Acidify and extract with ethyl ether; 100 cc., 50 cc., 50 cc.

Distil off ether and make up stock solution in ethyl alcohol. 1 rabbit unit = 6 to 8 mg. Yield 0.3 to 0.4 gm.

#### Ether fraction

#### Aqueous fraction (n)

Wash with  $NaHCO_3$ , HCl,  $H_2O$  as above

(Discard)

#### Ether fraction (o)

#### Washings (p)

Fatty acids as soaps  
(discard)

the solution again heated to boiling. The flask is again shaken vigorously and then cooled in the refrigerator for 12 hours. Filtration is carried out at ice box temperature. The majority of the precipitate, which is composed largely of neutral fat and cholesterol, remains in the flask. The entire process is repeated three more times so that none of the hormone remains adsorbed to the precipitate (*k*, discard). This procedure is technically easy and it removes practically all of the cholesterol and large amounts of neutral fat without much loss of the hormone. The 70 per cent methyl alcohol is removed by vacuum distillation and the residue (*j*) dissolved in absolute ethyl alcohol as a stock solution if further purification is not desired. Ordinarily 1 rabbit unit equals 30 to 40 mg.

The next step in purification is based upon the fact that ether solutions of the hormone can be washed with alkali without complete inactivation of the progesterin, even though in either aqueous or alcoholic solutions alkali promptly and completely destroys it. It was found that ethereal solutions of the hormone could be washed with 0.5 N alkali, thus removing the fatty acids present, and that this process removed practically all of the brown color. However, washing with 0.5 N alkali was not entirely satisfactory since some of the progesterin was always destroyed, so washing with aqueous  $\text{NaHCO}_3$  solution was employed. This causes only slight loss in potency and washes out almost as much inactive material as does 0.5 N  $\text{NaOH}$ .

This step is carried out as follows: The 70 per cent methyl alcohol solution (*j*) is reduced to about 15 to 20 cc. by vacuum distillation and the aqueous remainder is extracted four times with ethyl ether (100, 50, 50, and 50 cc.). The ether solutions are combined (*l*) and washed four times in a separatory funnel with saturated aqueous  $\text{NaHCO}_3$ , 20 cc. being used each time. The ether is then washed twice with water, twice with 0.2 N  $\text{HCl}$  (10 cc.), and then with water until the washings are neutral. All of the washings are combined, acidified with  $\text{HCl}$ , and extracted four times with ethyl ether (100, 50, 50, and 50 cc.). The ether solutions are combined and washed with  $\text{NaHCO}_3$  and  $\text{HCl}$  exactly as above, and the ether then combined with the ether fraction (*o*) from the first washings. The ether is distilled off and the residue dissolved in absolute ethyl alcohol. This preparation is a light-

yellow oil from which a crystalline substance separates upon being cooled to  $-4^{\circ}$ . 1 rabbit unit weighs 6 to 8 mg.

The subsequent purification of this oily end-product depends on the success with which the contaminating yellow oil can be removed. Two methods have been evolved, each of which is partially satisfactory, and each of which will give a colorless, crystalline product which is potent in about 0.6 mg. per day (*i.e.*, 1 rabbit unit = 3.0 mg.). This yellow oil is a fatty substance soluble in alcohol, ethyl ether, acetone, benzene, and to a less extent in petroleum ether. The hormone is soluble in hot petroleum ether (purified over  $\text{H}_2\text{SO}_4$  and redistilled, b.p.  $30-60^{\circ}$ ), as is some of the oil, but by repeated extraction of the oil with hot petroleum ether, evaporation of the ether, and reextraction, the process being repeated four or five times, a product is obtained which imparts no color to the petroleum ether and which precipitates out as a fine cloud immediately when the ether is allowed to cool down. If sufficient volume is used so that only a small amount precipitates out upon cooling to room temperature, and then the ether is allowed to evaporate very slowly at  $0^{\circ}$ , beautiful crystals are always obtained on the sides of the test-tube and in the solvent. In one lot, a crystal fully 2 mm. long was built up over a period of 3 days in this manner.

The second method depends on the fact that the hormone is soluble in hot dilute alcohol, whereas the majority of the yellow oil is not. The yellow oil is dissolved in absolute alcohol, heated to boiling, and then sufficient water is added to make the percentage 35 to 40. Immediately upon adding the water a milky cloud forms. The solution is again heated to boiling and then allowed to cool to room temperature. It is then placed in the ice box at  $-4^{\circ}$  for about 24 hours. Many feather-shaped crystals form during this process. The solution is then filtered cold, the filtrate being milky, and the white crystalline material is completely filtered out. The precipitate is washed through the filter with hot absolute alcohol and then ethyl ether. The solvents are evaporated off and the precipitate, which is nearly colorless, is again dissolved in hot alcohol, water added to make 40 per cent alcohol, and the freezing, precipitation, and filtration carried out as before. This somewhat unorthodox method of removing the oil is effective because the oil forms an emulsion which runs through

the filter whereas the hormone precipitates out in crystals which are large enough to be held back by the filter. Two such procedures suffice to remove all of the yellow oil from the white crystalline material. Both fractions however are potent, since complete separation of the hormone from the yellow oil has never been attained in any of the various preparations. The fact that the colorless crystalline product is potent in smaller dosage than the yellow oil makes it seem reasonable to suppose that the yellow oil is a contaminant. For example, one preparation weighing 500 mg. (1 rabbit unit = 10 mg. = 36 gm. of fresh tissue) was extracted with hot 37.5 per cent alcohol, repeatedly. The insoluble fraction gave a 3 + reaction with 30.0 mg. and the soluble fraction a 3 + reaction with 4.0 mg., and 62.5 per cent of the hormone was present in the soluble fraction. From the 37.5 per cent soluble fraction a crystalline substance active in 2.5 mg. dosage was prepared by freezing from about 10 cc. of 37.5 per cent alcohol. The recovery of the hormone in the crystalline fraction is not 100 per cent of that present in the crude oil. The equivalent of fresh tissue for a rabbit unit of the crystalline product is about 70 gm., whereas for the crude oil it is about 25 gm.; and curiously enough this loss of potency cannot be entirely recovered in the discarded fractions. The loss may be due, therefore, to deterioration of the active substance or to other factors as yet not fully understood. Thus far active crystalline material has been made from four different batches of tissue by one or the other of these methods.

A further method of final purification employed was high vacuum distillation. This was carried out on one extract which previous to distillation was active with a minimal dose of 8.7 mg. A fraction weighing 350 mg. (40 rabbit units) was then distilled at 0.002 mm. of Hg in a distilling apparatus which was designed and described by Hickman (1930) of the Eastman Kodak Company, and which uses a butyl thallate pump in place of the usual mercury vapor pump. The temperature was not accurately recorded since this was a preliminary experiment but the estimated temperature was about 150°. The distillate was collected in two fractions and the residue was saved as a third. Upon assay all fractions were potent. However, the second distillate fraction, which weighed 75 mg., was active in a 4 mg. dose and contained 25 rabbit units. This fraction contained, therefore, 62.5 per

cent of the total rabbit units and only 21.4 per cent of the total oil (Table IV). Furthermore the minimal dose in the other two fractions was 15 mg. The second distillate was a very pale yellow oil from which upon cooling a white crystalline substance readily separated. The procedure of high vacuum distillation resulted in no loss of activity and quite materially improved the purity of the product.<sup>2</sup>

Very little can be said regarding the chemical nature of progestin or even about the physical properties of the colorless crystalline product described above. Whether it is a chemical entity or a mixture remains to be seen, and in fact it may even be contaminated with estrin. We have known for a long time that the

TABLE IV  
*Yields from High Vacuum Distillation of Hormone at 0.002 Mm.*

Fraction	Yield	Dosage in mg. and resulting proliferation		Yield
	mg.			rabbit units
First distillate.....	70	++++ 28	<sup>0</sup> 5.0	5*
Second distillate.....	100	+++ 10	+++ 4	25
Residue.....	150	++++ 60	+++ 15	10

The + signs and the 0 above the figures have same significance as in Table I.

\* It is assumed that 14 mg. are 1 rabbit unit, since 28 mg. gave 5 + and 5 mg. 0.

purified progestin does not produce heat in rats unless given in fairly large doses and that even in the crude state 1 rabbit unit of progestin does not contain over 0.5 to 1 rat unit of estrin. One hesitates to attempt an accurate assay of progestin-containing extracts for their estrin content for the simple reason that it requires so much of the valuable extract to make such an assay.

Fels and Slotta (1931) have already announced the preparation of pure progestin in crystalline form but have not published, as yet, the particulars. Fevold and Hisaw (1932) have published

<sup>2</sup> I am indebted to Dr. K. C. D. Hickman of the Eastman Kodak Company for carrying out this high vacuum distillation.



more recently a preliminary note indicating that they have prepared a crystalline corporin (progesterin). The crystals are prepared by Fevold and Hisaw from the ether-soluble fraction of the extractives obtained by the acid-alcohol method of extraction and are readily soluble in alcohol, ether, acetone, or other similar solvents but much less soluble in water.

#### DISCUSSION

The recent history of the corpus luteum hormones, has, we fear, been rather confusing to the general reader, partly because of complexities inherent in the study and partly because of certain discrepancies regarding their solubilities and consequently the methods of separating them.

Hisaw and his coworkers discovered that the blood serum of pregnant rabbits contained a hormone which would relax the pelvic ligaments of the guinea pig, a condition which supervenes normally during the latter part of pregnancy in this animal, and that acid-alcohol extracts of corpora lutea would bring about the same result (Hisaw, 1929). This hormone was named relaxin and the purification was carried to such an extent that a crystalline product combined with sodium chloride was obtained and chemical analyses made (Fevold, Hisaw, and Meyer, 1930, c).

In 1929 we (Corner and Allen, 1929; Allen and Corner, 1929) described the preparation, by the extraction of corpora lutea with boiling alcohol, of a hormone which produced proliferation of the castrate rabbit's uterus, a reaction which also occurs during pregnancy, and which also maintained the embryos. This hormone was subsequently named progesterin (Allen, 1930, a). At first sight it seemed that both groups of workers were dealing with the same substance, one which produced numerous reactions, depending on the test used; but the product of Fevold, Hisaw, and Meyer (1930) was chemically different in that it was ether-insoluble and ours ether-soluble. Their crude preparation was also found to inhibit estrus in the rat, and to produce mucification of the vagina (Hisaw, Meyer, and Weichert, 1928) and to produce proliferation of the rabbit's uterus (Hisaw and Leonard, 1930), but they soon showed that the relaxin could be separated from the hormone responsible for the other reactions by the use of 99 per cent ethyl alcohol, the relaxin being insoluble and the second hormone (Hormone B,

or corporin) soluble (Fevold, Hisaw, and Meyer, 1930). In all of our work, however, on the chemical isolation of progestin (Allen, 1930, *a*) we adhered to the one test, *i.e.* the production of proliferation in the adult castrated rabbit, so we could not state at that time how many hormones our extracts contained. It was determined, however, that our purified preparations (1 rabbit unit = 3.0 mg.) did not produce relaxation (1930, unpublished observations).

The one fundamental difference between their procedure and that used by us is that their progestational substance (corporin, Hormone B) was found in the ether-insoluble fraction whereas our progestational substance (progestin) was found in the ether-soluble fraction. Specifically, they say that this hormone "is insoluble or very slightly soluble in ether, consequently the fats may be removed with no significant loss of hormone" (Fevold, Hisaw, and Meyer, 1930, *b*) and yet their method gave a yield of only 3 to 4 rabbit units per kilo, whereas our method gave yields 10 times as great. (This yield is deduced from the fact that it required the equivalent of 300 gm. of fresh tissue to produce a fair proliferation in 5 days (Hisaw and Leonard, 1930, Fig. 1). This is necessarily a deduction since they do not give yields for this method.) More recently, however, they (Fevold, Hisaw, and Leonard, 1932) have abandoned their previous method of separation, based on the supposed insolubility of the hormone in ether, and have adopted one similar in all its essentials to that published from this laboratory. By this new method they now obtain yields of 30 to 40 rabbit units per kilo, results which are in complete accord with those published from this laboratory (Allen, 1930, *a*, Table 1). It seems evident, therefore, that the preparation of crystals containing the active principle announced by them (Fevold and Hisaw, 1932) could scarcely have been accomplished without the use of ether (or some other organic lipid solvent) as a solvent for the hormone.

It also seems probable that the special mucifying factor which Fevold, Hisaw, and Leonard (1932) have separated from progestin and relaxin may not be a new hormone but estrin which has not been removed by their chemical methods of separation. This opinion is substantiated by the fact that Robson (1931) and Robson and Wiesner (1931) have shown that corpus luteum extracts

treated with alkali (and thus containing no progestin) and crude estrogenic extracts will produce mucification of the vagina in mice if subcornifying doses are given; and also by the fact that the author working jointly with Dr. R. K. Meyer (Meyer and Allen, 1932) has found that mucification can be produced in mice and guinea pigs with subcornifying doses of theelin (the crystalline estrogenic hormone isolated by Doisy). Since the reaction can be produced by theelin, it would seem necessary to prove that theelin does not produce this reaction, either directly or indirectly, or that theelin is contaminated by the mucifying hormone, before it can be demonstrated that a special mucifying hormone exists. Thus far no one has satisfied these postulates.

*Tests for Progestin*—The test which has been used in the assay of all fractions studied in the development of the above method has been the one described in detail previously by us (Corner and Allen, 1929); i.e., injection of the adult, recently castrated female rabbit for 5 days, with autopsy on the 6th day and study of histological sections of the uterus. This laborious procedure has been the only one which we have felt to be sufficiently reliable for use in the quantitative assays necessary to develop a trustworthy method of preparation of the hormone. The use of smaller rodents, such as rats and mice, although highly desirable from the standpoint of economy, has been found impractical because the changes in the endometrium of these animals are not sufficiently pronounced. Even immature rabbits cannot be used (Allen, 1930, b) unless they are given preliminary treatment with estrin so that their uteri may be brought into the adult state, since progestin apparently produces proliferation only in the uteri of those animals which have been under the influence of estrin. The production of mucification of the vagina of mice as was recently suggested by Harris and Newman (1931) also cannot be used as a specific test for progestin even though corpus luteum extracts will produce this reaction, since it has recently been shown that estrin is the hormone responsible for this change (Robson, 1931; Robson and Wiesner, 1931; Meyer and Allen, 1932).

Clauberg (1930), using corpus luteum extracts made by our method, has developed a method whereby the immature rabbit may be used instead of the adult, castrated rabbit. This, briefly, requires the use of non-castrated, immature females which have

been injected with 10 mouse units of menformone (estrin) daily for 8 days before the corpus luteum injections are begun. Corpus luteum extract is then injected for 5 days and the animal killed the next day. The uterus is submitted to microscopic study and the rabbit unit determined by much the same method as the one we use for the adult animal. We have never used the immature rabbit in our quantitative assays of potency, but we have found (Allen, 1930, b) that the immature rabbit if previously injected with estrin is as sensitive to progestin as the adult.

Knaus (1930) has developed a radically different test based on his observations that during the lutein phase of both pregnancy and pseudopregnancy the excised rabbit's uterus loses its *in vitro* response to the introduction of pituitrin into the bath. The same phenomenon of loss of response to pituitrin occurs if the rabbits are injected with progestin. (His extracts were made by our method (Corner and Allen, 1929).) These results obviously make possible a test based upon pituitrin response. His test consists in injecting the corpus luteum extract subcutaneously into the non-castrated adult female rabbit (which has been isolated for 3 weeks or more) four times at intervals of 12 hours, and then suspending the excised uterus in an oxygenated Ringer's solution. If the corpus luteum is potent and has been given in sufficiently large doses there is no contraction of the excised uterine muscle following the introduction of pituitrin into the bath. He has found that 0.5 gm. of crude oil injected into a 2 kilo rabbit is enough to cause the uterus to be completely refractory to pituitrin. That this inhibition is specific is shown by the fact that the extract does not prevent the response of the uterus to adrenalin or to quinine.\* We have never used this test as a criterion of potency chiefly because it is technically difficult and because the inhibition of pituitrin may possibly be due to some substance other than the one which causes proliferation of the endometrium, i.e. progestin.

Relaxation of the guinea pig's symphysis described by Hisaw (1929) as being producible by corpus luteum extracts need not be considered here since it has been shown (Fevold, Hisaw, and Meyer,

\* Knaus has studied in detail the pharmacodynamics of the uterus during the normal cycle, pseudopregnancy, and pregnancy. The references for this work are given by him in the papers referred to above.

1930, c) that this is due to a hormone distinctly different both chemically and physiologically from progesterin.

*Sources of Progesterin*—Thus far progesterin has only been prepared from corpora lutea. Other possible sources are, of course, the placenta, amniotic liquor, and the urine of pregnant women, since these have proved to be such potent sources of estrin (theelin, amniotin, menformone, and progynon). No one has succeeded in preparing progesterin from these sources, but this does not prove that none is present, because it has been shown by Courrier (1930), Leonard, Hisaw, and Fevold (1932), and Allen (1932) that estrin if given in large enough doses will prevent the occurrence of proliferation in the recently mated rabbit, and by several (Tausk, de Fremery, and Luchs, 1931; Robson and Illingworth, 1931; Leonard, Hisaw, and Fevold, 1932; and Allen, 1932) that estrin will prevent progesterin-containing extracts from having their characteristic proliferative effect on the rabbit's uterus. The fact that (Allen, 1932) 675 rat units of estrin completely inhibit 3 rabbit units of progesterin makes invalid any assay of urine, since 1 liter of urine usually contains about that much estrin. As a matter of fact, in this laboratory both urine and placenta have been extracted but they have been found devoid of any proliferative effect. If a method of removing the estrin can be developed then a negative result from these sources will have some significance.

*Oral Administration*—Englehardt (1932) has found that progesterin is ineffective by mouth and that it is destroyed by pancreatin. We have also found progesterin to be ineffective by mouth.

#### SUMMARY

An improved procedure for the preparation of highly purified progesterin is described and a brief discussion of the methods of assay is given.

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## THE METABOLISM OF SULFUR

### XIX. THE DISTRIBUTION OF URINARY SULFUR IN THE DOG AFTER THE ORAL ADMINISTRATION OF MONOBROMOBENZENE AS INFLUENCED BY THE CHARACTER OF THE DIETARY PROTEIN AND BY THE FEEDING OF L-CYSTINE AND DL-METHIONINE

By ABRAHAM WHITE AND HOWARD B. LEWIS

*(From the Department of Physiological Chemistry, Medical School, University  
of Michigan, Ann Arbor)*

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The synthesis of a phenylmercapturic acid after the administration of the monohalogen substitution products of benzene has been studied more extensively in the dog than in other animals (1). The most conclusive proof of this synthesis is to be obtained by isolation of the acid from the urine, but unfortunately this procedure is far from quantitative and isolation experiments give little information as to the extent of the synthesis. Since the sulfur of the molecule of the mercapturic acids is in organic combination, an approach to the quantitative aspect of this problem is available in the study of the partition of the urinary sulfur after the administration of the halogen derivative of benzene. If no compounds containing sulfur in unoxidized form other than the phenylmercapturic acid derivatives are excreted, such a study should give information of value; if other organic sulfur compounds are formed and excreted in the urine also, the evidence afforded by the sulfur partition studies must be interpreted with caution. Hele in experiments with dogs (1) and one of us (L.) in experiments with rabbits (2) have recently utilized this method of study. Other recent observers (3-7) have preferred for the most part to rely on the isolation of the mercapturic acid in the study of the problem.

It has generally been assumed that the amount of cystine available in the organism influences the extent of the synthesis of the mercapturic acids, but there exists some difference of opinion as



to whether cystine of endogenous origin may be utilized for purposes of synthesis as effectively as the cystine of the diet (either cystine liberated from protein in the processes of digestion or cystine fed as such). Thomas and his coworkers (3, 4) have held that the processes of endogenous protein catabolism do not result in the liberation of cystine or cysteine readily available for the formation of mercapturic acid after the administration of the monohalogen derivatives of benzene, since they were unable to isolate mercapturic acid from the urine of dogs fed bromobenzene and fasted or fed a protein-free diet, an observation confirmed by Muldoon, Shiple, and Sherwin (5). Abderhalden and Wertheimer (6) have criticized these experiments of Thomas because of the large amounts of bromobenzene fed to dogs weakened by prolonged fasting, and have obtained with shorter periods of fasting and smaller amounts of the halogen derivatives of benzene evidence of synthesis in fasted dogs, in fasted phlorhizinized dogs, and in dogs on a protein-free diet. They believe that the cystine requirements for reactions more essential than the detoxication of bromobenzene limit the extent of synthesis. The formation of phenylmercapturic acids in fasting rabbits has been demonstrated by Nishimura (7).

In most of the studies reported, the nature of the protein of the diet has had relatively little consideration. It appeared probable to us that more convincing evidence of the rôle of protein and of cystine in the synthesis of the mercapturic acids should be obtainable if the basal diet were adequate but of low protein content, particularly if the protein were so chosen that cystine was the limiting factor of the diet. We have studied the changes in the partition of urinary sulfur after the feeding of moderate amounts of bromobenzene to the dog in experiments in which casein and lactalbumin, as representative proteins of low and high content of cystine, supplied the protein element. We have also made similar studies in dogs fed a diet in which the protein was furnished by dried split peas, a diet known to be deficient in its cystine content (8), and have supplemented this diet by the addition of cystine. In view of recent observations (9, 10), which had indicated that methionine was able to replace cystine in the promotion of growth of white rats fed a diet in which an inadequate supply of cystine was the limiting factor, we have investigated the influence of this

other sulfur-containing amino acid on the mercapturic acid synthesis in the dog.

#### EXPERIMENTAL

Female dogs, accustomed to the routine of metabolism experiments, were used in these studies. The animals were kept in the usual metabolism cages and catheterized at regular 24 hour intervals. Immediately after catheterization, the daily food ration was given. The appetite of one of the dogs was little affected by the bromobenzene administration and for the most part, this animal consumed the diet regularly within 30 minutes after the

TABLE I  
*Composition of Basal Diets\**

	Diet A	Diet B	Diet C
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Casein.....	31.5		
Lactalbumin.....		29.0	
Dried peas.....			65.0
Sucrose.....	29.0	29.0	25.0
Lard.....	14.0	14.0	
Butter.....	14.0	14.0	25.0
Salt mixture (Karr)†.....	2.0	2.0	2.0
Tricalcium phosphate.....	4.0	4.0	4.0

\* The dog received 0.9 gm. of Yeast Vitamine-Harris Powder in a gelatin capsule (No. 00) daily.

† Hawk, P. B., and Bergeim, O., *Practical physiological chemistry*, Philadelphia, 10th edition, 695 (1931).

food was offered. The greater number of experiments were therefore conducted with this animal. On the few occasions when the dog exhibited a reluctance to eat, forced feeding was resorted to, a procedure easily and quantitatively carried out with this dog. All of the data presented in Tables II to V were obtained with this animal, a long haired mongrel bitch of about 9.5 kilos weight. Similarly planned experiments were carried out also with a second dog with results comparable to those presented.

The diets, recorded in Table I, supplied protein from three sources, commercial casein, a commercial purified lactalbumin, and split peas (green). Each of these materials was analyzed for

nitrogen and sulfur and each was examined also for the presence of inorganic sulfate sulfur. The protein of Diets A and B supplied 4.01 gm. of nitrogen, while the peas of Diet C furnished 2.64 gm. of nitrogen. The sulfur supplied by the protein foodstuffs of the three diets was 0.380, 0.424, and 0.140 gm. respectively. The commercial casein and the peas were found on analysis to contain inorganic sulfate sulfur in amounts which, when deducted from the total sulfur of the protein foodstuffs of Diets A and C, gave organic sulfur values of 0.274 and 0.092 gm. respectively for these diets. Cystine determinations on the peas by the Folin-Marenzi (11) method indicated 0.43 per cent of cystine. The daily ration of peas therefore supplied about 0.280 gm. of this amino acid. The calorific value of the diets was such that the dog received the equivalent of slightly over 50 calories per kilo of body weight.

In the preparation of Diet C, the peas as purchased were finely ground in a mill and sieved repeatedly to insure a uniform product. This powder was weighed out, mixed with 100 cc. of water, and heated on a steam bath for 30 minutes. The mixture was then cooled and the other ingredients of the diet were incorporated to give a uniform mixture. The yeast vitamin concentrate was fed separately from the rest of the diet in a small gelatin capsule (No. 00). The additional nitrogen and sulfur of the diet from the vitamin and capsule were 95 and 15 mg. respectively. Bromobenzene, cystine, glycine, and methionine were given likewise in gelatin capsules immediately after catheterization except in one series of experiments (Table III) in which it was desired to observe the effect of dividing the intake of cystine and administering it in smaller amounts at intervals.

The usual analytical methods were employed, the Kjeldahl-Gunning method for total nitrogen, the Folin colorimetric method for creatinine, and the gravimetric methods of Folin and of Benedict as modified by Denis for the partition of sulfur. Creatinine determinations were made as a check on the completeness of collection of the 24 hour specimens of urine; and since uniform values were obtained from day to day, the creatinine figures are not presented in the tables.

#### DISCUSSION

The data obtained in experiments in which casein and lactalbumin were fed as the protein components of the diet are presented

in Table II. Although casein does not differ significantly from many common proteins in its content of total sulfur, its cystine content is very low, 0.3 to 0.4 per cent (12). Lactalbumin, on the other hand, contains more cystine than any other of the common proteins except the keratins, values of from 2.60 (13) to 4.25 (14) per cent having been reported.

When casein was fed, the oral administration of moderate amounts of monobromobenzene resulted in a greater excretion of

TABLE II

*Excretion of Sulfur and Nitrogen after Oral Administration of Monobromobenzene As Influenced by Variation in Cystine Content of Protein of Diet*

The initial weight of the dog was 9.0 kilos; the final weight, 8.3 kilos. An interval of 11 days elapsed between the experiments recorded.

Experiment 1. Casein diet (Diet A)							Experiment 2. Lactalbumin diet (Diet B)					
Day	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S
	gm.	mg.	mg.	mg.	mg.	mg.	gm.	mg.	mg.	mg.	mg.	mg.
1	3.44	214	171	159	12	43	3.04	253	213	197	16	40
2	3.18	201	171	169	2	30	2.83	240	200	194	6	40
3	3.14	212	179	173	6	33	2.99	258	212	199	13	46
4	3.53	285	174	117	57	111*	3.15	264	162	118	44	102*
5	4.20	305	176	118	58	129*	3.41	290	161	117	44	129*
6	4.24	267	189	110	79	78*	3.55	284	142	97	45	142*
7	3.82	226	161	144	17	65	3.55	302	170	127	43	132*
8	3.55	230	180	169	11	50	3.33	270	189	162	27	81
9	3.21	222	178	165	13	43	3.26	260	203	193	10	57

\* The animal received daily 1 gm. of monobromobenzene orally.

ethereal sulfate sulfur, an excretion which increased progressively and reached its maximum value on the 3rd day of bromobenzene feeding. Associated with this increase in the urinary ethereal sulfates was a rise in the organic sulfur excretion, which fell sharply on the last day of the administration of the benzene derivative. Similar results were obtained in another experiment not detailed in the tables in which, on the days when bromobenzene was fed, the ethereal sulfate and organic sulfur fractions of the urine were 48, 49, and 64 and 102, 110, and 56 mg. respectively.

If the organic sulfur excretion is an accurate index of the extent of mercapturic acid synthesis, these experiments would indicate that on a low cystine diet, less cystine is available for synthesis after repeated feedings of bromobenzene and that less of the benzene derivative is excreted as the mercapturic acid and more in conjugation with sulfuric acid. In Experiment 1 (Table II) the average daily "extra" excretions for the 3 day bromobenzene period were 56 mg. of ethereal sulfate sulfur and 66 mg. of organic sulfur. When the protein element of the diet was supplied by lactalbumin (Experiment 2, Table II), a protein of greater cystine content, the excretion of ethereal sulfate sulfur was increased somewhat following the feeding of the bromobenzene, but no greater excretion was observed at the end of the bromobenzene period than at the beginning. The organic sulfur excretion rose to a higher level than when casein was fed and showed no tendency to decline with continued bromobenzene feeding as on the casein diet. Although the total extra sulfur excreted in response to the administration of bromobenzene was approximately the same as that observed on the casein diet, the distribution was different, more extra sulfur appearing in the organic sulfur fraction (an average of 84 mg. daily as compared with 66 mg.) and less as ethereal sulfate sulfur (32 and 56 mg. on the lactalbumin and casein diets respectively).

The effect of the administration of bromobenzene on the elimination of nitrogen was different in the two experiments. When casein was the source of protein, the toxicity of the bromobenzene, as evidenced by the increased nitrogen elimination, was greater than when the lactalbumin diet was fed. We believe that this difference in the nitrogen excretion is related to the amount of cystine supplied by the diet and available for conjugation. When dietary cystine is not available in adequate amounts, the protein of the tissues is catabolized to furnish cystine for mercapturic acid synthesis and the level of nitrogen excretion is increased. Further evidence in support of this view was obtained in experiments in which the casein diet was supplemented by cystine. In these experiments the distribution of urinary sulfur resembled that observed in the lactalbumin experiment; *i.e.*, a greater increase in the organic sulfur fraction and no change in the ethereal sulfate sulfur after the 1st day of the administration of bromobenzene.

The increase in nitrogen elimination was less marked in these studies also. Since more striking evidence of this supplementary action of cystine was obtained in experiments in which the basal diet was lower in sulfur content (Diet C), these studies of the supplementary effect of cystine added to the casein diet are not reported in detail in this paper.

Since the experiments with casein and lactalbumin had indicated that the type of protein fed influenced the urinary sulfur distribution and presumably the mercapturic acid synthesis after the administration of bromobenzene, attempts were made to devise a basal diet even lower in its sulfur content than the casein diet and low in cystine. The low sulfur content of the proteins of the pea has long been known (15) and later studies have shown that the value of these proteins in nutrition is limited by the deficiency of cystine (8). It was considered of interest, therefore, to determine the effect of the ingestion of bromobenzene when the protein of the diet was furnished by dried green peas (Diet C), a diet which, in some experiments, was supplemented by the addition of cystine. The amount of cystine fed was usually the equivalent of 1.5 atoms of sulfur per molecule of bromobenzene (*e.g.*, Experiment 3, Table III) and in one experiment the equivalent of 2 atoms of sulfur (Experiment 4, Table III). In the earlier experiments, the cystine was fed in a single dose at the same time as the benzene derivative. It seemed probable, however, that the absorption of bromobenzene proceeded more slowly than that of the cystine. In later experiments, in order to insure a supply of cystine during the period of active metabolism of the bromobenzene, the cystine was fed in divided doses. The initial feeding occurred at the time of the administration of the bromobenzene and the remainder was fed at 3 hour intervals as indicated in Table III. The details of two such experiments are presented in Table III.

It will be observed that the urinary elimination of sulfur with Diet C (Table III) is very much less than in those experiments in which the dietary proteins were casein and lactalbumin (Table II), the total sulfur approximating 100 mg. on most of the normal control days. In contrast to the marked differences between the excretions of total sulfur and total sulfate sulfur on control days in Tables II and III, no variations of the organic sulfur, associated

with character of the protein component of the diet, are evident. This uniformity offers a striking illustration of the constancy of

TABLE III

*Excretion of Sulfur and Nitrogen after Oral Administration of Monobromobenzene As Influenced by a Diet Low in Sulfur and Cystine Content (Diet C) with and without Added Cystine*

The initial weight of the dog was 8.4 kilos; the final weight, 8.35 kilos. An interval of 25 days elapsed between the two experiments. During this interval, the animal was fed the same standard diet (Diet C) as during the experiments.

Experiment 3							Experiment 4					
Day	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethe-real sulfate S	Or-ganic S	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethe-real sulfate S	Or-ganic S
	gm.	mg.	mg.	mg.	mg.	mg.	gm.	mg.	mg.	mg.	mg.	mg.
1	2.16	97	70	65	5	27	2.28	96	62	56	6	34
2	2.52	117	79	74	5	38	2.28	98	68	61	7	30
3	2.18	96	71	60	11	25	2.18	93	57	51	6	36
4	2.93	150	66	27	39	84*	2.95	153	76	29	47	77*
5	3.19	151	76	15	61	75*	3.01	133	45	6	39	88*
6	3.07	148	72	6	66	76*	3.13	142	60	2	58	82*
7	3.03	158	79	1	78	79*	3.37	162	70	0	70	92*
8	2.50	97	34	3	31	63	2.91	91	27	0	27	64
9	2.69	67	24	4	20	43	2.94	69	22	4	18	47
10	2.30	64	25	15	10	39	2.80	57	16	4	12	41
11	2.38	104	68	57	11	36	2.70	262	206	197	9	56†
12	2.30	226	126	80	46	100†	2.34	376	250	208	42	126§
13	2.04	290	167	127	40	123†	2.30	406	236	187	49	170§
14	2.16	315	178	119	59	137†	2.62	431	245	187	58	186§
15	2.21	346	183	124	59	163†	2.34	420	240	184	56	180§
16	2.41	185	100	75	25	86	2.25	144	67	47	20	77
17	2.35	100	62	53	9	38	2.25	114	64	51	13	50
18	2.38	100	66	56	10	34	2.28	103	63	53	10	40

\* The animal received daily 1 gm. of monobromobenzene orally.

† The animal received 1.5 gm. of cystine orally, given in three doses at 3 hour intervals.

‡ The animal received daily 1 gm. of monobromobenzene and 1.15 gm. of cystine orally. The cystine was given in two doses at 3 hour intervals.

§ The animal received daily 1 gm. of monobromobenzene and 1.5 gm. of cystine orally. The cystine was given in three doses at 3 hour intervals.

the organic sulfur of the urine notwithstanding the variations of the sulfur and cystine contents of the diets, and emphasizes anew the endogenous character of this sulfur fraction.

The effects of the administration of bromobenzene on the sulfur elimination when Diet C (Table III) was fed were similar to, although more striking than, the casein experiment already discussed (Experiment 1, Table II). A marked rise in the excretions of both the ethereal sulfate and organic sulfur fractions of the urine occurred. These increased excretions were accompanied by a marked decrease in the inorganic sulfate sulfur fraction, a decrease which became so marked with the continued administration of bromobenzene as to result in the practical disappearance of this fraction of urinary sulfur from the urine. This effect was particularly noticeable in Experiment 4 (Table III), in which neither the total sulfur nor the inorganic sulfate sulfur of the urine had returned to their normal values on the 3rd day after the bromobenzene period. As in the casein experiments (Table II), the excretion of the ethereal sulfate sulfur increased progressively as the administration of bromobenzene was continued, while the organic sulfur showed little change after the 1st experimental day. For the 4 day experimental periods when bromobenzene was fed, the extra ethereal sulfate sulfur excretions were 208 and 178 mg. in Experiments 3 and 4 respectively, while the extra organic sulfur eliminations in the same periods were 174 and 199 mg. respectively. Accompanying these changes in the sulfur distribution, in each experiment a marked rise in the excretion of total nitrogen occurred. Although the excretions of total sulfur and inorganic sulfate sulfur were much depressed immediately after the administration of the bromobenzene was discontinued, the nitrogen excretions remained above the normal level for several days. As a result, the urinary N:S ratios were unusually high, *e.g.* 32, 42, and 49 on days 8 to 10 of Experiment 4, indicating a marked retention of sulfur without a corresponding retention of nitrogen.

When cystine supplemented Diet C in the periods of bromobenzene administration, the results were striking. Most notable was the absence of any increased urinary nitrogen despite the addition of about 134 mg. of cystine nitrogen to the diet. Thus in Experiment 4, on the 4th day of bromobenzene feeding (day 7), the nitrogen excretion was 3.37 gm. or about 1.0 gm. above the normal level. In the same experiment, when cystine was fed with the bromobenzene, the nitrogen excretion was 2.34 gm. on



the 4th day of the experimental period, or approximately normal. In a similar experiment not recorded in detail, the nitrogen eliminations of the 4th days of the bromobenzene experimental periods were 2.48 and 3.62 gm. with and without the addition of cystine to the diet. In still another experiment with a different and larger dog, the corresponding nitrogen excretions were 3.47 and 4.53 gm. respectively. In each of our experiments in which cystine has been added to supplement the low sulfur, low cystine protein of the peas, the increased nitrogen catabolism which invariably occurred after the administration of the bromobenzene alone has been absent. We believe that this effect on the level of nitrogen metabolism is convincing evidence of the specific rôle of cystine in the detoxication of bromobenzene.

Further evidence of this rôle of cystine in influencing the metabolism of bromobenzene may be obtained from a study of the partition of sulfur in the cystine-bromobenzene experimental periods. In Experiment 3, the extra ethereal sulfate and organic sulfur excretions were 168 and 383 mg. respectively for the 4 day experimental period (days 12 to 15). These figures may be contrasted with the corresponding values of 208 and 174 mg. of the period in which bromobenzene without cystine was fed (days 4 to 7). It will be noted that the elimination of extra ethereal sulfate sulfur was not increased by the addition of cystine; in fact, a slight decrease, possibly within the error of the experiment, appeared. The extra organic sulfur excretion was more than doubled when cystine supplemented the diet in the bromobenzene periods.

In Experiment 4 (Table III), cystine equivalent to 2 atoms of sulfur per molecule of bromobenzene was fed, a preliminary feeding of cystine was given prior to the administration of the bromobenzene in order to insure if possible an excess of cystine or its catabolites in the tissues, and the cystine was divided into three doses administered at 3 hour intervals. In this 4 day experimental period (days 12 to 15), the extra ethereal sulfate sulfur of the urine was not increased as compared with the period of bromobenzene feeding without cystine (169 and 178 mg. for the 4 day period with and without addition of cystine to the diet). The extra organic sulfur, 522 mg., was more than double that excreted in a similar period of the administration of bromobenzene alone, 199 mg.

It was possible that some of this extra organic sulfur might have resulted from the administration of the cystine and have had no relation to the metabolism or detoxication of the bromobenzene. However, in control experiments with this same dog, in which cystine in amounts comparable to those of the preceding experiments was added to the basal diet (Diet C) in the absence of bromobenzene, we have never obtained an excretion of more than 60 mg. of extra organic sulfur in a 4 day period. Moreover, on those days in which cystine was fed in Experiments 3 and 4 and in other similar experiments, tests for cystine by the delicate cyanide-nitroprusside reaction in the urines have never indicated the presence of more than traces of the amino acid. While we regret the lack of an entirely satisfactory quantitative method for the determination of cystine in these urines, we consider it highly improbable that the high values for the extra organic sulfur in the cystine-bromobenzene periods are due to the presence of any significant amounts of cystine or the normal products of its catabolism.

In view of the interest in the recently discovered sulfur-containing amino acid of the protein molecule, methionine, and its possible importance as a supplement to or a substitute for cystine in nutrition (9, 10), we have studied the effects of the addition of *DL*-methionine (synthetic) to the basal pea diet (Diet C) on the urinary sulfur distribution when bromobenzene was fed. We have been unable to carry out as extensive studies as we have desired, since our supply of methionine was limited. We present, however, in Table IV the results of a series of such experiments with one of our animals. Since our knowledge of the fate of methionine in the dog was limited (15), methionine was fed without bromobenzene in a preliminary period (days 5 to 7). There occurred a marked depression of the elimination of nitrogen similar to that previously observed by one of us (16) when cystine was added to a diet low in its content of sulfur and of cystine. This low elimination of nitrogen continued on the 1st day after the feeding of methionine was discontinued. The excretion of organic sulfur increased somewhat and the values for this sulfur fraction remained above the normal for several days. Inasmuch as a more detailed study of the intermediary metabolism of methionine is in progress in this laboratory, we shall reserve further discussion of the possible significance of these findings until a later date.

When methionine (1.5 atoms of sulfur for each molecule of bromobenzene) was added to the basal diet in the periods in which

TABLE IV

*Excretion of Sulfur and Nitrogen after Oral Administration of Monobromobenzene As Influenced by a Diet Low in Sulfur and Cystine Content (Diet C) with and without Addition of Glycine and of dl-Methionine to Diet (Experiment 5)*

The initial weight of the dog was 11 kilos; the final weight, 10.7 kilos.

Day	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S	Day	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S
	gm.	mg.	mg.	mg.	mg.	mg.		gm.	mg.	mg.	mg.	mg.	mg.
1	2.24	113	77	63	14	36	21	2.23	157	110	99	11	47
2	2.28	108	72	57	15	36	22	2.16	151	110	100	10	41
3	2.28	106	79	68	11	27	23	2.63	208	124	90	34	84*
4	2.27	104	78	67	11	26	24	2.84	197	108	73	35	89*
5	2.09	192	146	134	12	46†	25	3.10	214	112	54	58	102*
6	1.81	271	225	218	7	46†	26	3.06	201	125	32	93	76*
7	1.86	297	236	230	6	61†	27	2.76	161	84	43	41	77
8	1.69	193	131	124	7	62	28	2.76	184	128	89	39	56
9	2.05	154	100	88	12	54	29	2.83	179	123	95	28	56
10	2.17	127	90	81	9	37	30	2.66	169	122	79	23	47
11	2.02	115	83	74	9	32	31	2.51	151	102	79	23	49
12	2.07	120	83	80	3	37	32	2.30	221	125	84	41	96†
13	2.02	113	76	72	4	37	33	1.81	284	154	104	50	130†
14	1.83	215	118	83	35	97†	34	1.63	293	154	101	53	139†
15	1.62	279	157	121	36	122†	35	1.67	311	168	110	58	143†
16	1.46	268	145	106	39	123†	36	1.74	187	106	81	25	81
17	1.76	304	162	117	45	142†	37	2.29	154	101	85	16	53
18	1.66	190	115	85	30	75	38	2.39	149	102	93	9	47
19	2.18	146	90	71	19	56	39	2.31	149	104	90	14	45
20	2.25	150	102	91	11	48							

\* The animal received daily 0.72 gm. of glycine and 1 gm. of monobromobenzene orally.

† The animal received daily 1.4 gm. of dl-methionine orally.

‡ The animal received daily 1.4 gm. of dl-methionine and 1 gm. of monobromobenzene orally.

bromobenzene was fed (days 14 to 17 and days 32 to 35), the results resembled those of the experiments<sup>1</sup> in which cystine supplemented

<sup>1</sup> Further evidence of the similarity in the action of cystine and methionine in the detoxication of bromobenzene is to be found in the symptoms

the basal diet during bromobenzene administration (Table III). There was a marked depression of nitrogen excretion, the elimination of ethereal sulfate sulfur increased little after the initial rise of the 1st day of bromobenzene feeding, and there was an increased excretion of organic sulfur which reached its maximum value on the last day of the feeding period. The effects of methionine feeding were so remarkable that the question arose as to whether the changes observed were specific for the sulfur-containing amino acids or whether they might be due to the influence of any amino acid regardless of its sulfur content. As a further control, we administered glycine with the bromobenzene in amounts comparable to those of the sulfur-containing amino acids (days 23 to 26, Table IV). When glycine was fed, the sulfur and nitrogen excretions were quite comparable to those in the experiments in which bromobenzene was fed without the addition of an amino acid (Table III). The nitrogen excretion was increased; the ethereal sulfate sulfur increased progressively, reaching its maximum value on the 4th day of the bromobenzene period, while the rise in organic sulfur was less marked and the excretion declined sharply on the last day of the period. The nitrogen excretion during the initial control period of 4 days was 9.08 gm., a level which was maintained approximately throughout subsequent control periods. In the two periods in which methionine and bromobenzene were fed together, the nitrogen excretions during 4 days were 6.67 and 7.41 gm., respectively, while in the period in which bromobenzene and glycine were fed, the nitrogen elimination was 11.63 gm. These differences in nitrogen are striking and indicate that methionine administered with bromobenzene is as effective in preventing an increased level of nitrogen catabolism as is cystine.

The interpretation of the changes in organic sulfur is not easy. There occurred an unquestionably greater increase in the organic sulfur fraction of the urine when methionine was fed with bromobenzene than when glycine and bromobenzene were fed. The feeding of methionine alone resulted in some increase in the organic sul-

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exhibited after the feeding of the bromobenzene. When the basal diet was fed alone or supplemented by glycine, the animal was depressed for several hours after the administration of the bromobenzene; when methionine or cystine was added to the basal diet, the toxic effect of the bromobenzene was clearly less marked.

fur of the urine. We do not believe, however, that the higher values for organic sulfur in the methionine-bromobenzene periods as compared with the similar values in the glycine-bromobenzene period can be explained entirely as a result of the ingestion of the methionine. The increases in extra organic sulfur are similar to those observed in the cystine experiments of Table III and other like experiments, although probably not so marked.

As stated previously, the changes in the organic sulfur fraction of the urine after the administration of monohalogen derivatives of benzene have been considered as quantitative evidence of the extent of synthesis of the phenylmercapturic acids. While we believe that such an interpretation of the data is probably justifiable, we hesitate to apply it to our own results and to conclude that the ingestion of methionine leads to a more extensive synthesis of a mercapturic acid or to the synthesis of some similar substance in which the methionine molecule or a product of its metabolism is concerned. Such a conclusion can be justified only when satisfactory methods for the quantitative determination of the mercapturic acids are available for the analysis of the urines after the feeding of benzene derivatives or when a product derived from the conjugation of methionine, or a derivative of this amino acid, with monobromobenzene shall have been isolated. For the present, it is sufficient to point out the similarity in the changes in the sulfur metabolism when either cystine or methionine is administered with bromobenzene and to suggest that methionine may function as does cystine in the detoxication of the monohalogen derivatives of benzene. It is possible also that cystine and methionine may have some common product of intermediary metabolism which is essential for the normal function of the organism and that when methionine is supplied by the diet, the cystine present is thereby made available for the detoxication of the benzene derivatives.

Certain observations made in connection with these experiments are of interest in relation to the problem of the origin and significance of the increased urinary ethereal sulfates after bromobenzene feeding. It has been pointed out that the level of ethereal sulfate excretion after the administration of bromobenzene appeared to be related to the amount of cystine available in the organism for the detoxication of the monohalogen benzene deriva-

tive. Whenever bromobenzene was given to the animal maintained on a dietary régime low in cystine, the excretion of ethereal sulfates increased progressively with the repeated daily doses of the toxic compound. In contrast to these results, when adequate cystine was supplied, either as the free amino acid or in the form of protein (lactalbumin), the ethereal sulfate excretion tended to rise to a level above the normal excretion but remained approximately at that level during the days of the feeding of monobromobenzene.

In view of these results, it appeared probable to us that whenever adequate amounts of cystine were available, the animal organism detoxified monobromobenzene by conjugation with the amino acid and excreted it as the bromophenylmercapturic acid. If a cystine deficiency existed, the animal was forced to resort to some other means of removing the toxic bromobenzene. A portion of the compound might be excreted as such and a portion might be either partially or completely oxidized. The first step likely to occur in the oxidation of bromobenzene would result in the formation of *p*-bromophenol. That the latter reaction may take place in the animal organism is not at all improbable. Under conditions of stress, drastic oxidations may occur in the animal organism, as is exemplified in the experiments of Jaffé (17), who was able to isolate small amounts of muconic acid from the urine of dogs which had been given benzene. This evidence for the cleavage of the benzene ring *in vivo* would support a hypothesis which assumes the much milder reaction; that is, the introduction of hydroxyl groups into the benzene nucleus. Conditions which favor the formation of these phenolic compounds should, therefore, augment the urinary excretion of ethereal sulfates. It is significant that throughout these experiments progressively increasing excretions of ethereal sulfates were noted when bromobenzene was administered to the animal maintained on a low cystine diet. An oxidation of bromobenzene to *p*-bromophenol, occurring when a cystine deficiency prevents detoxication of the halogen benzene derivative by the formation of a mercapturic acid, would increase the excretion of ethereal sulfates.

Further experimental evidence is presented in Table V, in which are given the details of an experiment in which *p*-bromophenol was fed with and without the addition of sodium sulfate to Diet C.

The administration of *p*-bromophenol resulted in an increased excretion of sulfur as ethereal sulfates and a marked decrease in the inorganic sulfate fraction, a decrease so marked as to result

TABLE V

*Excretion of Sulfur and Nitrogen after Oral Administration of p-Bromophenol As Influenced by a Diet Low in Sulfur and Cystine Content (Diet C) with and without Feeding of Sodium Sulfate (Experiment 6)*

The initial weight of the dog was 10.7 kilos; the final weight, 10.0 kilos.

Day	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S	Day	Total N	Total S	Total sulfate S	Inorganic sulfate S	Ethereal sulfate S	Organic S
	gm.	mg.	mg.	mg.	mg.	mg.		gm.	mg.	mg.	mg.	mg.	mg.
1	2.57	107	69	61	8	38	17	2.46	337	310	217	93	27*
2	2.54	109	78	71	7	31	18	2.35	378	350	249	101	28*
3	2.35	106	75	66	9	31	19	2.56	381	354	215	139	27†
4	2.53	105	77	71	6	28	20	2.45	365	338	192	146	27†
5	2.67	142	110	8	102	32†	21	2.34	384	357	222	135	27†
6	2.70	133	105	0	105	28†	22	2.10	97	70	61	9	27
7	2.72	134	105	0	105	29†	23	2.11	94	67	63	4	27
8	2.67	139	110	0	110	29†	24	2.04	98	71	63	8	27
9	2.51	48	21	0	21	27	25	2.09	98	72	63	9	26
10	2.53	46	22	11	11	24	26	2.45	140	117	6	111	23§
11	2.34	113	87	81	6	26	27	2.32	137	115	0	115	22§
12	2.35	109	76	68	8	33	28	2.32	138	110	0	110	23§
13	2.25	112	81	73	8	31	29	2.51	110	86	0	86	24§
14	2.31	289	261	258	3	28	30	1.93	37	14	2	12	23
15	2.40	296	267	259	8	29	31	2.04	49	24	14	10	25
16	2.46	380	352	254	98	28*	32	1.96	115	90	80	10	25
							33	2.04	99	74	64	10	25

\* The animal received daily 1.22 gm. of sodium sulfate and 1.1 gm. of *p*-bromophenol orally.

† The animal received daily 1.22 gm. of sodium sulfate and 1.65 gm. of *p*-bromophenol orally.

‡ The animal received daily 1.1 gm. of *p*-bromophenol orally.

§ The animal received daily 1.65 gm. of *p*-bromophenol orally.

|| On days 14 and 15 the animal received 0.81 and 1.22 gm. respectively of sodium sulfate orally.

in the complete disappearance of this fraction of urinary sulfur. Further suggestive evidence that ethereal sulfates were being formed at the expense of inorganic sulfate sulfur during the period of the administration of the *p*-bromophenol was obtained

in experiments in which sodium sulfate was fed with the phenol. During those experimental days (days 19 to 22), there was a slightly increased excretion of ethereal sulfates as compared with the excretion when bromophenol was fed alone with the basal diet (days 26 to 29). The administration of the *p*-bromophenol did not lead to any alteration in the organic sulfur of the urine. Hence the suggestion that oxidation to the phenol is a preliminary step in the synthesis of *p*-bromophenylmercapturic acid after the administration of bromobenzene appears improbable. Further studies of this problem are in progress.

#### SUMMARY

1. The distribution of sulfur in the urine after the oral administration of monobromobenzene has been studied in dogs maintained on diets, the protein elements of which varied in their content of sulfur and of cystine.

2. Greater increases in the organic sulfur fraction of the urine after bromobenzene feeding were observed when the protein of the diet was furnished by lactalbumin, a protein rich in cystine, than when the protein element was supplied by casein or by peas, both low in cystine content. On these low cystine basal diets, the extra ethereal sulfate sulfur excretion after bromobenzene was greater than when a basal diet of higher cystine content (lactalbumin) was fed.

3. The addition of either *l*-cystine or *dl*-methionine to the low-cystine basal diet (peas) in the bromobenzene feeding periods prevented the increased nitrogen excretion in the urine which occurred after the administration of bromobenzene when the basal diet was fed without supplement. The sulfur distribution resembled that found in the experiments in which lactalbumin was the source of the dietary protein.

4. The effects of cystine and methionine on the nitrogen elimination and on the distribution of the urinary sulfur following bromobenzene feeding appeared to be specific since similar results were not obtained when glycine was fed with bromobenzene.

5. The relation of these changes in sulfur metabolism to the problem of the synthesis of the phenylmercapturic acids is discussed.

6. No increase in the organic sulfur of the urine was observed



to result from the feeding of *p*-bromophenol under conditions of a low cystine basal diet (peas). This is believed to support the theory of Coombs and Hele (18), who have suggested that two paths of catabolism of bromobenzene are open, the one the oxidation, conjugation, and elimination as ethereal sulfates, the other the synthesis of mercapturic acid. It is considered probable that if abundant cystine is available, the major portion of the bromobenzene is conjugated with the amino acid and excreted as the mercapturic acid, while, if the supply of cystine is limited, oxidation, conjugation, and elimination of the bromobenzene as ethereal sulfates is the predominant means of detoxifying the benzene derivative.

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## THE CALCIUM AND PHOSPHORUS CONTENT OF THE BRAIN IN EXPERIMENTAL RICKETS AND TETANY

By ALFRED F. HESS, JOSEPH GROSS, MILDRED WEINSTOCK, AND  
FRIEDA S. BERLINER

*(From the Department of Pathology, College of Physicians and Surgeons,  
Columbia University, New York)*

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In the course of a metabolism study of rickets, in which various tissues of the rat were analyzed for total calcium and phosphorus, it was noted that whereas the amounts of these substances were approximately normal in the kidneys, lungs, skin, and other soft tissues of the body, the percentage of calcium in the brain was decidedly lower than in the normal animal. There are no figures in the literature for the content of calcium and phosphorus in the brain of normal rats. Recently we have carried out many analyses of this kind, some of which are reproduced in Table I, and which show that calcium in the brain amounts to about 182 mg. and total phosphorus to about 1278 mg. per 100 gm. of dried weight; the variations in calcium ranged from about 100 to 260 mg. and in phosphorus from about 1000 to 1400 mg. For these analyses a modification of the Fiske and Logan method (1) was employed, two brains being used for each determination. They were placed in 95 per cent alcohol for 3 days and dried to constant weight. The method was followed up to and including the precipitation of calcium as oxalate. From this point, instead of filtering through a filtration tube, and then converting to calcium oxide and finally titrating with sodium hydroxide, the calcium was filtered through a Gooch crucible with an asbestos mat. (The asbestos had been treated with  $H_2SO_4$  and  $KMnO_4$ .) The calcium oxalate was then titrated with  $KMnO_4$ . The filtrate obtained after filtering off calcium oxalate was precipitated for phosphorus as ammonium phosphomolybdate.

It may be added that the content of calcium in the brain of

various animals seems to differ remarkably. It has been stated that the brain of the dog as well as that of the human being contains about 20 mg., and that of the rabbit about 510 mg. per 100 gm.

Table II gives the figures for calcium and total phosphorus in the brain of rats in which rickets had been induced. It will be noted that these figures are distinguished by a low percentage of calcium; in other words, whereas the normal brain has an average content of about 182 mg., only 59 mg. were found in the rachitic

TABLE I  
*Calcium and Phosphorus in Brain of Normal Rats*

No. of rats	Weight of rats	Normal diet	Days on diet	Serum		Brain	
				Ca	Inorganic P	Ca	Total P
	gm.			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.
2	55-113	Sherman Diet D	24	11.7	10.3	254.1	1289.3
2	54-107			11.9	9.1	138.6	1279.3
2	48- 93		23	10.8	9.3	254.9	1340.9
2	48- 87			10.9	8.9	107.4	1350.0
2	57- 89		20	10.9	9.4	100.6	1468.1
2	57- 92			11.2	10.1	100.5	1212.5
3	65- 94	Sherman Diet B Bills stock diet	10	13.0	7.7	202.5	988.0
1	50- 94			10.1	7.0	264.0	972.6
2	69-124		17	10.6	8.2	137.3	1463.3
2	60-102			10.7	8.4	225.6	1513.1
4	60-164		58	11.2	9.2	250.0	1148.4
1	175-202		28			184.8	1186.4
1	120-145		16			191.1	1347.6
1	128-165					145.6	1334.3
Average.....						182.6	1278.1

rats, the lowest figure being 37 mg. and the highest 76 mg. The total phosphorus of both groups was about the same. A reduction of the calcium content of the brain was found, irrespective of the nature of the rickets-inducing ration, three standard rations being used for this purpose (2-4).

Having met with this unexpected result, we altered the rickets-inducing diets somewhat in order to ascertain, if possible, the nature of the factor which led to withdrawal of calcium from the

brain. In the first place, a ration was constituted which by the addition of  $\text{Na}_2\text{CO}_3$  was brought to the same degree of alkalinity as the McCollum Diet 3143 (2) but was normal in all other respects. It will be noted in Table III that this alkaline ration induced no lowering of the calcium in the brain such as was always found in rickets. A second ration, comparatively low in its calcium content, was formed by incorporating grains with

TABLE II  
*Calcium and Phosphorus in Brain of Rachitic Rats*

No. of rats	Weight of rats	Rachitic diet	Days on diet	Roentgenographic rickets	Serum		Brain	
					Ca	Inorganic P	Ca	Total P
	gm.				mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.
2	54-67	McCollum Diet 3143	24	Moderate	10.4	2.4	76.7	1429.6
2	65-80			"	10.8	3.4	72.8	1451.1
2	45-54		21	"	11.0	3.5	45.4	1109.0
1	44-54			Slight			68.1	1268.0
2	52-50		22	Marked			62.3	1295.8
3	72-70	Sherman-Pappenheimer Diet 84		"	9.7	4.0	46.9	1404.5
2	55-85		28	"	10.4	4.7	77.5	1274.0
2	62-89			"	10.9	2.8	60.6	1406.8
2	44-50		25	Moderate	11.4	3.2	36.9	1258.0
2	55-72	Steenbock Ration 2965	25	"	11.4	2.7	43.4	1245.0
Average.....							59.0	1314.2

dried milk, the ratio of Ca:P being 0.8:1. Table III shows that with such a diet no change was brought about in the brain, the calcium figures being 221 and 346 mg. per 100 gm. of dried tissue. In a third test the ratio of Ca:P was markedly decreased by using the standard McCollum ration prepared without the usual addition of  $\text{CaCO}_3$ ; this had a Ca:P ratio of only 1:10. The type of rickets which is occasioned by a low calcium, high phosphorus ration was induced in these rats. Under these conditions the calcium was found to have been markedly reduced, only 33 to 43 mg. being found, so that it must be concluded that the calcium of

the brain can be lowered, not only by the usual rickets-inducing ration, high in calcium and low in phosphorus, but by one which is low in calcium and high in phosphorus.

As a matter of course, tests were carried out to ascertain whether the calcium in the brain could be brought back to the

TABLE III

*Calcium and Phosphorus in Brain of Rats Fed on Diet Markedly Alkaline or Low in Calcium*

No. of rats	Weight of rats	Diet	Roentgeno-graphic rickets	Serum		Brain		
				Ca	Inor-ganic P	Ca	Total P	Inor-ganic P
	gm.			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.	mg. per 1000 gm.
2	57- 89	Sherman Diet D	None	10.9	9.4	100.6	1468.1	
2	57- 92	(20 days)	"	11.2	10.1	100.5	1212.5	
2	57- 95		"	10.8	9.3			922.8
2	47- 77		"	10.7	9.8			840.0
2	45- 91		"	10.3	9.7			984.4
1	40- 90	3.18 per cent	"	8.5	10.1	130.0	1348.6	
2	63-101	Na <sub>2</sub> CO <sub>3</sub> added	"	12.2	10.1	137.4	1383.1	
2	40- 79	(20 days)	"	10.4	10.7	104.5	1381.3	
2	52- 77		"	10.9	9.7			720.4
2	57- 91		"	10.8	8.7			856.3
1	40-130	Grains and dry	"	8.7	9.5	221.7	968.0	
3	45-132	milk* (58 days)	"	8.4	9.8	346.2	1285.0	
2	47- 78	McCollum Diet 3143	Slight	7.3	9.3	32.8	1243.4	
		without CaCO <sub>3</sub> †						
2	42- 65	(21 days)	"	6.7	8.2	42.7	1134.1	

\* Ca:P ratio, 0.8:1.

† Ca:P ratio, 1:10.

normal level by means of various antirachitic agents. Table IV gives a review of this experiment. Ultra-violet irradiation with the mercury vapor lamp was carried out daily for a period of 10 days, the lamp being placed at a distance of 1 foot and exposures given for periods of 12 minutes. This intensity of irradiation was insufficient to induce a definite rise of calcium in the brain. A similar failure was encountered when cod liver oil was given for the same period in the dosage of 1 drop of a super D preparation,

which had a potency  $2\frac{1}{2}$  times that of standard oil. In all of these animals healing of the epiphyses was evident in radiographs. Irradiated ergosterol (viosterol) when given for 15 days, 0.025 cc. daily, was likewise without effect on the brain. It will be noted that Table IV contains figures representing not only the total but also the inorganic phosphorus of the brain. This aspect will be discussed later. It may be mentioned, however, in this

TABLE IV

*Calcium and Phosphorus in Brain of Rachitic Rats Treated for a Short Period with Antirachitic Agents*

No. of rats	Weight of rats gm.	Roentgenographic rickets*	Antirachitic agent	Healing shown by Roentgenograph	Serum		Brain		
					Ca	Inorganic P	Ca	Total P	Inorganic P
					mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.	mg. per 1000 gm.
2	73-68	Moderate	Ultra-violet rays (10 days)	Moderate	11.2	6.7	54.4	1304.6	
3	65-63	Marked		"			48.5	1252.5	
2	59-54	Moderate	Cod liver oil (10 days)	Slight					487.1
2	62-62	Marked		Moderate	11.9	5.7	67.1	1426.6	
2	69-65	"		"			40.5	1417.3	
2	60-51	Moderate		"					546.1
2	55-57	"		"					515.0
2	64-62	Marked		"					570.6
2	51	Moderate	Viosterol (15 days)	Marked	11.8	5.7	79.8	1404.6	
2	67	"		"			52.3	1350.0	

\* McCollum Diet 3143.

connection that there was no change either in phosphorus or calcium as the result of these various antirachitic measures.

Rickets was likewise treated by substituting an adequate ration for the McCollum ration. Recently we showed that, "Tetany can be induced in rachitic rats simply by an abrupt change from a rickets-inducing ration, high in calcium and low in phosphorus, to a normal ration of dried milk, or of dried milk and whole wheat. The fall in calcium in the serum which is brought about by this means, develops within 48 hours but is maintained for only a few days" (5). Table V gives the results of an experiment in which

tetany, as manifested by low calcium in the serum, was induced by this means. It shows that the calcium of the brain was approximately the same as had been found in rats suffering from uncomplicated rickets; if anything, there was a tendency for the calcium to be lower and the total phosphorus somewhat higher. In some instances the normal Sherman Diet D (6) was given for 21 days and by this means complete healing was brought about in the bones and the inorganic phosphorus of the blood restored to its normal level. By this shift the calcium in the brain was raised definitely

TABLE V

*Calcium and Phosphorus in Brain of Rachitic Rats in Which Tetany Was Induced by Shift to Normal Diet*

No. of rats	Weight of rats <i>gm.</i>	Roent- genographic rickets*	Diet	Healing shown by Roentgeno- graph	Serum		Brain	
					Ca	Inorganic P	Ca	Total P
					<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>
2	44-102	Moderate	Sherman Diet	Complete	11.6	9.2	96.5	1387.0
2	40-105	"	D (21 days)	"	11.2	10.4	83.6	1452.7
2	46- 52	"	Same (3 days)	Moderate	7.7	10.7	68.3	1533.9
2	65- 93	"		"	11.2	12.3	56.2	1491.4
2	52- 64	Marked		"	7.4	11.0	45.0	1385.9
2	64- 95	"		Marked	6.7	13.7	55.9	1203.2
3	59- 60	"	Sherman Diet B (4 days)	Moderate	6.1	9.5	62.3	1086.5

\* McCollum Diet 3143.

above the rachitic level, although the test period did not suffice to restore it to the normal concentration. It would seem that restoration of calcium in the brain comes about slowly.

An experiment carried out to ascertain how quickly calcium is withdrawn from the brain of rats receiving a rickets-inducing diet, showed that after a period of only 6 days, when merely slight rickets had been induced, the brain had been robbed somewhat of its calcium, two of the animals having only 91 mg. and two others only 77 mg. per 100 gm. of dried tissue. It should be added that

it was found that in this instance the McCollum ration, instead of having the established Ca:P ratio of about 4.0:1 had the ratio of 6:1.<sup>1</sup>

Parathyroidectomy was carried out in a series of rats, some of which were on a normal (Bills stock diet (8); Sherman Diet D (6)) and others on a rickets-inducing diet. In the former, the calcium in the brain was normal in amount 3 days after the operation, at a time when the concentration of calcium in the serum was below 7 mg. per 100 cc. of blood. This was also true of two animals which

TABLE VI  
*Calcium and Phosphorus in Brain of Parathyroidectomized Rats*

No. of rats	Weight of rats	Diet	Days on diet	Days after operation	Serum		Brain	
					Ca	Inorganic P	Ca	Total P
	gm.				mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.
2	55-91	Sherman Diet D	10	3	7.6	10.1	195.9	1176.5
2	57-94				7.6	11.7	261.8	1447.3
3	74-98				6.4	11.6	174.9	1307.5
2	37-89				8.0	12.9	101.1	1109.7
2	70-105				6.4	14.6		1352.4
1	180-164	Bills stock diet	28	25	6.3	11.3	289.4	1338.4
1	200-200						343.0	1278.8
1	120-178				6.1	16.2	188.8	1286.7
1	150-180				7.0	11.0	215.1	1401.2
2	58-66	McCollum Diet 3143*	24	3	11.2	2.7	75.2	1369.7
2	58-67				10.7	3.9	78.1	1374.9

\* Rickets shown by Roentgenogram.

were not killed until 25 and 71 days, respectively, after operation and in which the serum contained approximately 6 mg. of calcium and the inorganic phosphorus was abnormally high (Table VI).

<sup>1</sup> A few years ago we called attention to the fact that the ratio of Ca:P in a diet may vary greatly according to whether yellow corn or yellow cornmeal is used as an ingredient (7). Since this time we have found that whole ground corn may vary in its content of P from 63 to 265 mg. per 100 gm. Gelatin, another constituent of rickets-inducing diets, has been found to contain from 266 to 417 mg. of Ca and 40 to 253 mg. of P. These variabilities are important and should be considered and obviated in the standardization of rations for quantitative tests of antirachitic agents.



On the other hand, in rachitic rats on which parathyroidectomy was performed the calcium in the brain was about 75mg. This low concentration is of special interest in view of the fact that on this high calcium diet calcium in the serum remained normal in spite of the parathyroids having been removed. It is evident that *there is no relationship between the calcium content of the brain and that of the serum.* In dogs which had been subjected to parathyroidectomy, Dixon and his colleagues (9) found the calcium and phosphorus of the muscles to be unchanged in amount, and Cooke

TABLE VII  
*Calcium and Phosphorus in Brain of Rats Injected with Parathormone*

No. of rats	Weight of rats	Diet	Parathormone injected daily (3 wks.)	Roentgenographic rickets	Serum		Brain	
					Ca	Inorganic P	Ca	Total P
	gm.		cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 gm.
2	51-116	Sherman Diet D	0.25	None	11.0	8.7	103.8	1389.1
2	49- 93		None	"	10.8	9.3	149.0	1355.3
2	48- 93			"	10.9	9.4	254.9	1340.9
2	48- 87			"	10.9	9.0	107.4	1350.0
2	51- 50	McCollum Diet 3143	0.5	Moderate	9.4	3.4	47.1	1103.5
1	48- 62		0.25	"	10.6	2.5	57.8	1130.0
2	41- 54			Marked	10.9	3.3	45.0	1222.0
2	41- 49			Slight	10.4	3.4	54.4	1204.0
2	44- 51		None	Moderate	11.5	3.2	43.6	1214.4
2	45- 54			"	11.0	3.6	45.4	1109.0
1	44- 54			Slight			68.1	1268.0

reported (10) that in dogs which had undergone a similar operation and were dying of tetany the calcium in the brain was slightly above the normal.

Some tests were made to ascertain what effect the injection of parathormone would have on the calcium of the brain, as it seemed possible that the diminution of calcium in this organ might be due to a decreased function of the parathyroid glands. In one set of rats rickets was induced and in another a normal diet was given. In the former, in spite of daily injections of 0.5 cc. of parathormone the calcium of the brain fell to the rachitic level; in the latter it remained unchanged (Table VII).

The natural inference was that, in experimental rickets, calcium is withdrawn from the brain as calcium phosphate. In order to

TABLE VIII  
*Inorganic Phosphorus in Brain of Rachitic and Normal Rats*

Final weight of rats*	Diet	Days on diet	Roentgenographic rickets	Serum		Brain Inorganic P		
				Ca	Inorganic P			
gm.				mg. per 100 cc.	mg. per 100 cc.	mg. per 1000 gm.		
37	McCollum Diet 3143	42	Marked	10.6	5.1	404.3		
51				10.2	6.1	345.7		
59		31		9.7	4.0	470.6		
68				9.7	4.0	493.9		
51		21	Moderate			376.5		
54						669.8		
45						348.4		
55				10.2	5.7	541.6		
53						516.6		
57						546.3		
66				Marked		600.5		
67						525.0		
63					10.3	5.8	529.6	
73						469.3		
60				Moderate		498.4		
Average.....						474.9		
95	Sherman Diet B	21	None	10.8	9.3	922.8		
77				10.7	9.8	840.0		
91				10.3	9.6	984.4		
122	Bills stock diet	42				717.0		
124						709.4		
115				11.5	8.9	980.0		
86						888.4		
124						651.0		
112						719.2		
108					11.8	8.3	739.6	
125							703.8	
Average.....						805.1		

\* Average weight of the two rats used for each analysis.

test this hypothesis, estimations of the inorganic phosphorus in the brain were carried out. To this end the method of Fiske and Subbarow (11) was used with the following slight modifications.

Two brains were used for each determination; they were removed as soon as possible, weighed in a centrifuge tube containing cold trichloroacetic acid, and frozen in a mixture composed of ice and salt. They were ground immediately, washed back into the tube, and made up to 15 cc. For analysis 2 cc. were used. Moisture content was noted in the brains of rats of the same group in which Ca and P were determined. The total phosphorus of the rat brain averages about 1200 mg. per 100 gm. and of this total amount about 80 mg. are inorganic phosphorus. It was found (Table VIII) that in rickets, whereas the total phosphorus is not appreciably reduced, the inorganic phosphorus undergoes a diminution parallel to that of the calcium. The average figure was about 47.5 mg.; in other words, about half the normal. Figures for inorganic phosphorus of the brain are set down also in Table IV, which shows that after cod liver oil had been given for a 10 day period the inorganic phosphorus was still low.

#### DISCUSSION

The fact that stands out clearly from this series of tests is that rickets in the rat is accompanied by a definite and marked decrease of calcium in the brain. However, the relationship of calcium in the brain to experimental tetany is less sharply defined. In the earliest experiments in this field, those by MacCallum and Voegtlin (12) which were carried out on dogs, a diminution in calcium of the brain was found following parathyroidectomy. This may have been due to a deficiency of calcium in the diet, for as stated, Cooke's experience in regard to tetany brought about in dogs in a similar manner was just the contrary. In rats we have failed to find a direct relationship between tetany brought about by parathyroidectomy and a decrease of calcium in the brain. For example, in a rat weighing 172 gm., which died in convulsions  $\frac{1}{2}$  hour after parathyroidectomy, the calcium in the brain was found to be 295 mg., and in another which died 4 days after operation and manifested marked tremors during this interval the calcium was 343 mg. To our mind, it is the rickets rather than the tetany which leads to withdrawal of calcium from the brain.

When we turn to the relationship between the calcium content of the brain and its concentration in the serum, it is evident that the one is independent of the other. The very fact that the total

calcium of the serum is normal in rickets and that this is the very condition which leads to the marked diminution of calcium in the brain, makes further consideration of this aspect unnecessary.

The diminution of calcium in the brain was not caused by a lack of calcium in the diet, but developed when large amounts were fed and its ratio to phosphorus was 4:1. If, however, the calcium in the diet was markedly reduced, the phosphorus content remaining normal, the percentage of calcium in the brain was brought down to a very low level.

Although we have made no study of the calcium content of the brain of human beings, a few words may be added in regard to this vexed question. In 1905 Quest (13) reported that he found the calcium low in the brains of infants who had suffered from tetany. However, Quest's material is open to question and his conclusions, although confirmed by some, have been strongly controverted by others. In order to elucidate this problem, a sharp distinction will have to be drawn between analyses of the brains of infants who had preliminary rickets and those exceptional cases in which the tetany was primary. Possibly the chemical alterations in the brain which accompany rickets are the basis of infantile tetany and this explains the well known observation that rickets almost always precedes tetany. This suggestion must be regarded simply as a hypothesis, to be substantiated or disproved by further investigations on human material.

#### SUMMARY

Rickets in the rat is associated with a marked decrease of total calcium in the brain. The percentage of inorganic phosphate is similarly decreased. These changes come about in spite of the fact that the diet is high in calcium. The return to normal levels is slow.

There is no relationship between the concentration of calcium in the blood and its concentration in the brain. In rickets the total calcium is normal in the blood and diminished in the brain; in parathyroid tetany it is low in the blood and undiminished in the brain.

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## THE ANTINEURITIC VITAMIN

### IV. THE PREPARATION OF A HIGHLY POTENT CONCENTRATE\*

BY RICHARD J. BLOCK† AND GEORGE R. COWGILL

*(From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York, and the Laboratory of Physiological Chemistry, Yale University, New Haven)*

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The antineuritic vitamin appears to react similarly to the better known organic bases (1). As a base, it should be soluble in organic solvents; as a salt it should be insoluble in these. We hoped that this principle would permit a considerable degree of concentration with very little loss of vitamin. A preliminary note on the success of these experiments was contained in our previous communication (2). More than thirty experiments have been carried out in this connection and the results assayed for vitamin contents by the weight maintenance, preventive, and curative techniques (3); we believe that the data so obtained can be quantitatively evaluated.

#### EXPERIMENTAL

*Starting Material*—We have used a wide variety of vitamin sources without noting any significant difference in the final potency of our concentrates.

The starting materials have been: (a) a crude aqueous extract of rice polishings;<sup>1</sup> (b) a concentrate of rice polishings prepared by adsorption on and removal from fullers' earth;<sup>1</sup> (c) the aqueous extract (a) after it had been treated by the carbon tetrachloride-oxidation technique (2); (d) the concentrate (b) after being purified

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† Honorary Fellow in Physiological Chemistry, Yale University, 1931-32.

<sup>1</sup> We are indebted to Eli Lilly and Company, Indianapolis, for the preparation of this material.

TABLE I  
*Extraction of Vitamin with Ether*

Experiment No.*	Kind of test†	Alkali	pH of extraction‡	Pigeon units per cc. organic solvent		N per pigeon unit	Recovery (approximate)	Concentration	Comments
				Starting material	Recovery				
						mg.	per cent	×	
III C1	M	NaOH	R.	0.40	0.16	2.2	40	250	Starting material
III C5a	M-C					0.008			"
IV D	"					2.3			"
IV D4a	M-P	"	"	0.19	0.17	0.03	85	70	
IV D4c	P	"	"	0.19	0.16	0.02	80	100	
IV D4n	C	"	"	0.75	0.14	0.14	10	15	
IV D4o	"	"	"	0.75	0.13	0.06	15	15	
V A1	M					0.80			Starting material
V A7	P	"	P.	1.0	0.17	0.007	25	100	
V A8	P-C	"	R.-P.	2.0	0.20	0.007	50	100	Ether + C <sub>6</sub> H <sub>5</sub> COCl
V A9	"	"	"	2.0	0.17	0.009	25	100	" + C <sub>6</sub> H <sub>5</sub> SO <sub>2</sub> Cl
V A10	P	"	"	2.0	0.09	0.010	10	20	" + CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> Cl
V A11	"	Ba(OH) <sub>2</sub>	"	2.0	0.17	0.037	25	20	
V A12	"	"	"	2.0	0.17	0.050	25	20	" + C <sub>6</sub> H <sub>5</sub> COCl
V A13	"	"	"	2.0	0.15	0.014	25	50	" + C <sub>6</sub> H <sub>5</sub> SO <sub>2</sub> Cl
V A14	"	"	"	2.0	0.09	0.020	10	40	" + CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> Cl
V A15	M	Na <sub>2</sub> CO <sub>3</sub>	R.	2.0	0.19	0.090	10	10	
V A16	C	"	"	2.0	0.10	0.030	10	30	" + C <sub>6</sub> H <sub>5</sub> COCl
V A17	"	"	"	2.0	0.10	0.060	10	15	" + C <sub>6</sub> H <sub>5</sub> SO <sub>2</sub> Cl
V A18	P	"	"	2.0	0.10	0.060	10	15	" + CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> Cl

VI A1	M	NaOH	R.	0.80	0.20	2.3	25	75	Starting material
VI A2	P	"	"	0.80	0.13	0.030	20	150	Ether + C <sub>6</sub> H <sub>5</sub> COCl
VI A7	M	"	"	0.80	0.09	0.015	15	65	" + C <sub>6</sub> H <sub>5</sub> SO <sub>2</sub> Cl
VI A9	"	"	P.	0.80	0.09	0.036	15	100	" + CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> SO <sub>2</sub> Cl
VI A10	M-P	"	"	0.80	0.09	0.020	15		
I T1	P-G	"	R.			0.010			

\* All experiments that seemed unusually significant were duplicated with essentially the same results.

† M = weight maintenance, P = prevention of polyneuritis, G = curative test.

‡ Color of phenolphthalein paper. R., red; P., purple.



by the oxidation procedure (2); (e) a yeast concentrate prepared in a manner similar to that described in (b);<sup>1</sup> (f) activated fullers' earth from yeast;<sup>1</sup> (g) an aqueous extract of dried yeast;<sup>2</sup> (h) an aqueous solution of tikitiki extract;<sup>3</sup> (i) an aqueous suspension of wheat bran.

*Types of Alkali*—In order to liberate the vitamin as the "free base," the pH of the aqueous solution was adjusted between the ranges pH 8.5 to 14 by means of the following alkalies:  $\text{Na}_2\text{CO}_3$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{Ba}(\text{OH})_2$ ,  $\text{NaOH}$ , and  $\text{KOH}$ . The results given in Table I seem to indicate that within these ranges there is no appreciable difference in the amount of vitamin extracted. However, it appears that more nitrogenous impurities are extracted at higher alkalinities; therefore, until this point has been investigated further, we advise extraction from a solution of about pH 9 to 10.

*Solvent*—The following solvents have been used: ethyl ether, petroleum ether, benzene, toluene, methyl acetate, ethyl acetate, isoamyl acetate, tertiary amyl alcohol, *n*-butyl alcohol, isobutyl alcohol, and ethylene dichloride. Table I gives the results with ethyl ether as the solvent. This substance was employed most extensively because its physical properties make it advantageous for general laboratory use. The solvents mentioned above may be divided into three classes according to the ease with which they extract the vitamin. These are: (Class A) ethyl ether, ethyl acetate, *n*-butyl alcohol, tertiary amyl alcohol; (Class B) petroleum ether, toluene, methyl acetate; (Class C) benzene, isoamyl acetate, isobutyl alcohol, and ethylene dichloride.

In the course of a large number of experiments, we found that the most important factor in the extraction was neither the potency of the original material, the ratio of the amount of water to the organic solvent, the alkali, the pH of the aqueous solution, nor the organic solvent employed, but it was *the amount of solvent used per pigeon unit* present in the starting material. It required 5 to 10 cc. of the Class A solvents, 8 to 16 cc. of Class B, and more than 15 cc. of Class C, to extract 1 pigeon unit of vitamin. Tables I and II give some of the experimental results upon which we base this conclusion. In many of the experiments, the ether solutions of the vitamin were washed with widely varying amounts of dilute

<sup>1</sup> From the Northwestern Yeast Company.

<sup>2</sup> From the Philippine Bureau of Sciences, Manila.

alkali without any detectable change in the potency of the vitamin in the solvent. Therefore, we may tentatively conclude that, in the specific case of ether, the vitamin has a true solubility under the conditions employed. The studies with the other solvents have not been extensive enough to warrant any conclusions.

*Acid*—The acids which were used to neutralize the vitamin "base" were hydrochloric, sulfuric, acetic, lactic, tartaric, and citric.

TABLE II

*Extraction of Vitamin with Various Organic Solvents*

In each case NaOH was the alkali used for titration; the phenolphthalein paper was red.

Experiment No.*	Kind of test†	Solvent	Pigeon units per cc. organic solvent		N per pigeon unit	Recov-ery (ap-proxi-mate)	Con-centra-tion
			Start-ing mate-rial	Recov-ery			
					mg.	per cent	×
IV D4b	M-P	Butyl alcohol-ether	0.19	0.17	0.090	85	25
IV D4d	P	Toluene	0.19	0.08	0.10	45	20
IV D4e	M	Petroleum ether	0.19	0.13	0.04	70	50
IV D4f	"	Ethyl acetate	0.19	0.16	0.04	90	50
IV D4g	P	Methyl acetate	0.19	0.10	0.15	45	15
IV D4h	M	Isoamyl acetate	0.19	0.06	0.30	30	10
IV D4i	P	Tertiary amyl alcohol	0.19	0.17	0.15	90	15
IV D4j	M	Butyl alcohol	0.19	0.17	0.10	90	25
IV D4k	P	Isobutyl alcohol	0.19	0.05	0.5	25	4

\* All experiments that seemed unusually significant were duplicated with essentially the same results.

† M = weight maintenance, P = prevention of polyneuritis, C = curative test.

*Preparation of a Potent Vitamin B<sub>1</sub> Concentrate*—Although it is apparent from the above discussion that a great many modifications in the technique are possible as long as a few fundamental principles are adhered to, a description of a typical experiment might be helpful.

600 cc. of an aqueous extract of rice polishings containing 350 mg. of total solids, 4.2 mg. of nitrogen, and 2 pigeon units per cc. were cooled to 0°.

Dry  $K_2CO_3$  was added (165 gm.) to make the resulting solution alkaline to about pH 10. The ice-cold alkaline solution was then extracted with ether in an apparatus similar to the one described in (4). The extraction flask was placed in a freezing mixture in order to maintain the temperature of the alkaline vitamin solution below  $5^\circ$ . The receiver contained sufficient acid to neutralize the vitamin, as well as any other basic substances which might be extracted. The apparatus was so arranged that in the course of 4 hours 7 cc. of ether per pigeon unit passed through the vitamin solution. The rice polishings extract was removed by suction and, a little water having been added, the excess ether was distilled into the extraction bottle. The vitamin solution was cooled in an ice bath, filtered, and made up to desired volume.

In such an experiment one should recover 80 to 95 per cent of the total potency of the starting material. By this procedure, impurities estimated as total solids are reduced about 500 times, while the nitrogenous impurities are decreased about 200 times. A rough estimate (1) indicates that about 10 per cent of the total nitrogen present in this concentrate is vitamin nitrogen.

When only small amounts of the vitamin are required, say 50 pigeon units,<sup>4</sup> the extraction can be carried out just as well in a separatory funnel. In this technique the ether can be washed with very dilute alkali before the vitamin is removed by extraction with acidulated water, and therefore results in a slightly purer concentrate.

An attempt to remove any substances which would react with aromatic acid chlorides in the presence of alkali was carried out as follows:

To the combined ether extracts, after washing with dilute alkali in a separatory funnel, an excess of one of the following reagents was added: benzoyl chloride, benzene sulfone chloride, and *p*-toluene sulfone chloride. The ether solution was shaken and allowed to stand for 5 minutes, after which the vitamin was removed by shaking against dilute hydrochloric acid. This procedure was unsuccessful as shown by the fact that the amount of nitrogenous impurities remaining in the vitamin concentrate was not reduced.

<sup>4</sup> 1 pigeon unit equals about 10 Sherman-Spohn rat units or 2.5 Chick-Roscoe units.

*Outline of Essential Steps*

1. *Starting Material*—Any source of the vitamin.
2. *Alkali*—Any common alkali capable of adjusting the pH from 8.5 to 14.
3. *Solvent*—A common organic solvent immiscible with water.
4. *Acid*—Any non-oxidizing or non-reducing organic or inorganic acid.
5. *Purity of Concentrate*—This depends on the starting material, solvent, and pH of extraction.
6. *Yield*—This is directly proportional to the amount of organic solvent used per pigeon unit present in the starting material.
7. *Losses*—(a) Alkaline destruction: decreased by low temperatures. (b) Oxidative: care must be taken in removing the ether from the acidified concentrate. (c) Mechanical.

## SUMMARY

A method is described for purifying the antineuritic vitamin, based on the theory that it is an organic base and as such should be capable of extraction from aqueous alkaline solutions by organic solvents. By this technique, vitamin B<sub>1</sub> concentrates are obtained directly from rice polishings or yeast, which approximate in potency the concentrates obtained by the Jansen and Donath procedure up to and including the platinum stage. The yields obtained by our method are approximately 90 per cent, whereas those obtained by the more elaborate procedure are about 5 per cent.

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# METABOLIC CHANGES INVOLVING PHOSPHORUS AND CARBOHYDRATE IN THE AUTOLYZING GASTROCNEMIUS AND CARDIAC MUSCLES OF NORMAL, OF THYROIDINIZED, AND OF ADRENALECTOMIZED ANIMALS\*

BY MARY V. BUELL, MARGARET B. STRAUSS, AND  
E. COWLES ANDRUS

*(From the Cardiographic Laboratory and Chemical Division, Department of  
Medicine, the Johns Hopkins Hospital and the Johns Hopkins  
University, Baltimore)*

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## INTRODUCTION

In spite of the many fundamental contributions made during the past 5 years to our knowledge of the chemical nature of those muscle constituents which contain P, few attempts have been made to relate these compounds quantitatively either with each other or with the process of glycolysis.

For discussion of the present status of our knowledge of various aspects of the chemistry of muscle the reader is referred to recent review articles (1-7). The new fundamental ideas initiated by the discovery of phosphocreatine by Fiske and Subbarow (8) have necessitated a thorough revision of previous theories of glycolysis, the older literature being almost meaningless in the light of the new findings. The chemical events taking place in the living animal when muscle contracts are extremely complex and are reversible in the sense that during relaxation the muscle is restored approximately to its original condition. To focus attention upon anaerobic glycolysis *per se* we have chosen to study the changes in autolyzing isolated muscle tissue because (1) the secondary influence of other tissues, hormones, etc., is excluded, (2) the reactions are essentially anaerobic and irrever-

\* This investigation was financed in part by a grant from the Bingham Fund.

sible (defined as above), and (3) the process takes place comparatively slowly. Most modern studies of muscle autolysis have centered around the decomposition of labile organic compounds of P, when incubated in bicarbonate solution. Interest in autolysis under these conditions was stimulated by Embden and Zimmermann's (9) finding in 1924 that the inorganic P of muscle was greatly increased under these circumstances, a fact which they mistakenly interpreted as a quantitative manifestation of the decomposition of lactacidogen.

Extensive clinical and pathological studies have been made of those conditions which are characterized by muscle weakness, such as hyperthyroidism and Addison's disease. An analysis of the chemical abnormality in the light of the newer knowledge has not been previously attempted. In this paper are reported the results of a study of the effects of thyroxine and of adrenal insufficiency upon the chemical composition of autolyzing *skeletal* muscle, which indicate that lactic acid production under these conditions is much less than in normal muscle. A similar survey has been made of the chemical composition of the autolyzing *cardiac* muscle of normal and of thyroxinized animals, which indicates that thyroxine does not bring about a depression of lactic acid formation in this tissue.

That cardiac muscle shares in the physiological effects of thyroxine has been repeatedly demonstrated (10-13). Moreover, it has been shown that this effect survives the removal and isolation of the heart and persists for hours thereafter.

#### EXPERIMENTAL

*Removal of Muscle*—The gastrocnemii were removed from living animals (cats and rabbits, anesthetized by the intraperitoneal injection of sodium amytal) under conditions designed to give resting values. The muscle was freed by blunt dissection around the Achilles tendon, the tendon was cut, and the muscle was quickly excised and thrust into liquid air. Not more than 4 to 5 seconds elapsed between the time of cutting the tendon and immersion in liquid air. In spite of this speed certain values obtained on analysis were not strictly resting, when compared with others in the literature, owing to the fact that autolytic

changes take place very rapidly during the time required to freeze so large a piece of muscle.<sup>1</sup>

Before the heart was removed the animal was anesthetized with sodium amytal, artificial respiration was instituted, the thorax opened, and the pericardium slit. The heart was lifted up by the apex and was rapidly cut out, leaving behind most of the auricles with the large blood vessels, fat, etc. The ventricles were quickly blotted with filter paper to remove adherent blood, and were thrust into liquid air. For the extensive chemical determinations undertaken either two or three hearts were required. These were pulverized and mixed while frozen.

*Preparation of Filtrates*—The frozen muscle, cardiac or skeletal, was pulverized, mixed, and sampled. Appropriate quantities (3 gm.) were placed in large tared weighing bottles, containing 7 cc. of 0.9 per cent NaCl, which were rapidly reweighed to the nearest mg. The chloride solution served to keep the muscle moist and to render conditions approximately anaerobic. The muscle was allowed to autolyze at room temperature for periods of 0, 5, 10, 20, and 40 minutes. Each sample was then treated with 5 per cent trichloroacetic acid, making the final dilution 1:10 on the assumption that the water content of muscle was 80 per cent (in actual determinations it was found to be slightly lower). The whole muscle mixture was thoroughly ground in a mortar with acid-washed sand and filtered immediately through acid-washed paper. Filtrates were clear and remained so on standing.

*Methods*—The following methods were used for the analyses: for inorganic P and total acid-soluble P, the Fiske and Subbarow (14) method; for phosphocreatine, the indirect method of Fiske and Subbarow (15); for Embden ester, the method of Cori and Cori (16); for pyrophosphate, the Lohmann method (17); for adenylic acid, the method of Buell and Perkins (18) especially adapted for this purpose; for lactic acid, the method of Friedemann and Kendall (19), with West's (20) improved apparatus; for glycogen the Pflüger (21) method, with certain modifications, essentially those of Osterberg (22). The final determination of

<sup>1</sup> Recognizing this source of error, we have continued to use this technique because large samples of muscle were essential for the extensive analyses undertaken.



TABLE I

*Autolyzing Gastrocnemius of Normal Animals*

All results are expressed as milli-equivalents, either of P or of potential lactic acid.

Experiment No.	Autolysis <i>min.</i>	Lactic acid	Glycogen as lactic acid	Isolated Embden ester fraction					Ortho inorganic P	Phosphocreatine P	Adenylic pyrophosphates		Total acid-soluble P	
				Hexose as lactic acid	Calculated ester P	Free nucleotide P	Total organic P	Extra organic P			Pyroinorganic P	Adenylic acid P	Determined total P	Calculated total P
1	0	14.6	115	9.0	4.5		4.5		18.8	12.8	9.4	4.7	55.2	50.2
	5	21.8	47	11.5	5.7		9.4		29.4		7.4	3.7	54.0	49.9
	10	41.6	28	10.7	5.3		14.0		32.7		2.0	1.0	53.0	49.7
	20	55.8	16	7.9	3.9		9.9		36.2		1.5	0.7	51.6	48.3
	40	71.4	11	7.7	3.8		11.6		36.9		0.0	0.0	50.5	48.5
2	0	7.6	80	7.4	3.7		3.5		15.7	11.9	16.1	8.1	57.8	55.3
	5	13.9	58	8.0	4.0		5.6		22.6	7.7	11.3	5.7	57.8	52.9
	10	25.0		9.6	4.8		10.3		35.8	0.0	4.7	2.4	57.8	53.2
	20	49.8	28	8.2	4.1		11.3		39.3		2.1	1.1	59.6	53.8
	40	75.1	27	7.7	3.9		10.9		42.1		0.0	0.0	57.8	53.0
3	0	7.7		10.4	5.2		6.3		18.4	10.5	11.9	6.0	53.0	53.1
	5	16.2		10.1	5.1		8.7		30.0	2.8	6.7	3.4	53.8	51.6
	10	23.1		10.8	5.4		10.5		34.9		5.5	2.8	54.9	53.7
	15	39.3		13.4	6.7		13.2		36.2		4.1	2.1	55.7	55.6
4	0			16.1	8.1	0.9	9.6	0.6	13.0	7.9	18.4	9.2	59.1	58.1
	30			8.9	4.5	5.0	9.7	0.2	40.8		1.3	0.7	59.1	52.5
	30-A			7.9	4.0	9.6	13.4	-0.2	41.4		1.4	0.7	65.1	56.9
5	0			7.9	4.0	0.8	3.8	-1.0						
	10			9.1	4.6	1.1	5.3	-0.4						
	20			8.7	4.4	1.7	7.8	1.7						
	40			7.1	3.6	2.1	7.7	2.0						
6	0	10.4		13.7	6.9		9.5		10.5	10.9	14.1	7.1	52.3	52.1
	5	22.2	56						19.8	4.7	10.0	5.0	54.1	
	10	30.0	11	16.5	8.3		12.1		27.0		7.9	4.0	53.0	51.0
	20	51.0	11	10.7	5.4		11.3		34.9		2.9	1.5	54.9	50.6
	40	71.7	6	11.2	5.6		12.3		39.7		1.7	0.9	57.3	54.6

*Experiment 1*—October 15, 1931. Cat.

*Experiment 2*—November 2, 1931. Cat.

*Experiment 3*—October 5, 1931. Cat.

*Experiment 4*—January 26, 1932. Cat. To Sample 30-A 5 milli-equivalents of nucleotide P were added for a recovery experiment. Excellent recovery was noted.

*Experiment 5*—May 11, 1932. Cat.

*Experiment 6*—December 1, 1931. Rabbit.

TABLE II

*Autolyzing Gastrocnemius of Thyroxinized Animals*

All results are expressed as milli-equivalents, either of P or of potential lactic acid.

Experiment No.	Autolysis <i>min.</i>	Lactic acid	Glycogen as lactic acid	Isolated Embden ester fraction					Ortho-inorganic P	Phosphocreatine P	Adenylic pyrophosphate		Total acid-soluble P	
				Hexose as lactic acid	Calculated ester P	Free nucleotide P	Total organic P	Extra organic P			Pyro-nucleic P	Adenylic acid P	Determined total P	Calculated total P
1	0	4.8	40	5.8	2.9		5.5		10.3	13.5	13.6	6.8	49.0	49.7
	5	8.6	21	6.1	3.1		6.7				10.4	5.2	48.4	
	10	16.7		5.4	2.7		8.0		31.0	0.0	3.6	1.8	50.9	44.4
	20	20.3	10	4.4	2.2		8.7		38.7		2.1	1.1	51.6	50.6
	40	21.2	8	4.1	2.1		9.4		41.9		0.4	0.2	53.4	51.9
2	0	5.6	48	6.8	3.4	0.7	6.4	2.3	15.3	6.6	15.2	7.6	50.6	51.1
	5	10.1	23	6.2	3.1	1.6	8.6	3.9	22.9	4.3	4.7	2.4	47.2	42.9
	10	15.4	10	5.2	2.6	1.6	12.3	8.1	31.8		3.0	1.5	48.9	48.6
	20	15.3	10						35.4		1.0	0.5	49.0	
3	0	5.4	21	9.2	4.6	1.2	6.1	0.3	15.0	6.2	10.6	5.3	44.5	43.2
	5	10.2	17	9.2	4.6	3.8	8.2	-0.2	29.7	0.0	1.2	0.6		39.7
	10	10.9		4.8	2.4		7.3		31.1		1.3	0.7	45.5	40.4
	20	14.0	16	6.0	3.0	2.2	9.6	1.4	34.7		0.0	0.0		44.3
	40		11	5.4	2.7		7.8		34.6		0.0	0.0	46.6	42.4
4	0	6.5	78	9.0	4.5	0.8	7.0	1.7	14.3	14.3	14.0	7.0	54.5	56.6
	5	13.9	35	9.9	5.0	1.9	9.5	2.6	23.0	5.0	5.5	2.8		45.8
	10		15	12.2	6.1	2.9	12.8	3.8	31.9		3.7	1.9	54.9	50.3
	20	34.5	15	6.8	3.4	4.6	10.7	2.7	36.9		1.8	0.9	54.5	50.3
5	0	7.1	73	9.4	4.7	0.6	4.8	-0.5	8.1	8.4	13.4	6.7	43.5	41.4
	5	12.1	18	10.5	5.3	0.8	5.8	-0.3	12.4	4.2	10.0	5.0	41.8	37.4
	15	24.4	9	10.4	5.2	1.2	8.0	1.6	21.0		2.4	1.2	37.0	32.6
	25	36.7	8	8.5	4.3	1.8	8.7	2.6	34.3		0.8	0.4	47.2	44.2

*Experiment 1*—December 18, 1931. Cat. 23 mg. of thyroxine; 35 per cent loss in weight.

*Experiment 2*—December 21, 1931. Cat. 17.3 mg. of thyroxine; 30 per cent loss in weight.

*Experiment 3*—January 6, 1932. Cat. 6 mg. of thyroxine; 26 per cent loss in weight.

*Experiment 4*—January 13, 1932. Cat. 53 mg. of thyroxine; 13 per cent loss in weight.

*Experiment 5*—May 3, 1932. Rabbit. 1.9 mg. of thyroxine; 14 per cent loss in weight.

glucose was made by the method of Hagedorn and Jensen (23). Wherever possible the technique was checked by repeated analyses of standard solutions.

TABLE III  
*Autolyzing Gastrocnemius of Adrenalectomized Animals*

All results are expressed as milli-equivalents, either of P or of potential lactic acid.

Experiment No.	Autolysis <i>min.</i>	Lactic acid	Glycogen as lactic acid	Isolated Embden ester fraction					Ortho-inorganic P	Phosphocreatine P	Adenylic pyrophosphate		Total acid-soluble P	
				Hexose as lactic acid	Calculated ester P	Free nucleotide P	Total organic P	Extra organic P			Pyrophosphoric P	Adenylic acid P	Determined total P	Calculated total P
1	0	4.2	72	7.0	3.5	0.7	3.7	-0.5	11.5	10.3	19.0	9.5	50.0	54.0
	5	7.8	32	7.9	4.0	1.9	5.7	-0.2					53.0	
	10	19.5	13	9.0	4.5	3.0	8.5	1.0	32.0	0.0	4.7	2.4	50.9	47.6
	30	29.7	9	6.5	3.3	3.7	8.5	1.5	38.5		1.2	0.6	50.0	48.8
	30-A	29.2							31.3				52.0	
2	0	3.2	61	5.5	2.8	0.6	3.1	-0.3	5.2	6.0	27.4	13.7	47.2	55.4
	5	9.7	49	6.5	3.3	1.3	5.6	1.0	21.3	4.6	12.1	6.1	50.3	49.7
	10	24.0		6.5	3.3	1.6	7.2	2.3	26.1	1.8	7.2	3.6	45.5	45.9
	20	31.3	17	5.9	3.0	2.5	8.2	2.7	39.8		2.0	1.0	52.3	51.0
	40	30.8	14	5.1	2.6	2.2	7.2	2.4	39.8		0.7	0.4	49.3	48.1
3	0	4.4	76	9.6	4.8	0.6	4.0	-1.4	10.6	13.3	15.5	7.8	50.0	51.2
	5	9.3	58	9.8	4.9	0.9	5.3	-0.5	18.9	5.9	13.0	6.5	49.3	49.6
	10	18.8	54	13.0	6.5	1.8	8.9	0.6						
	20	33.9	14	9.8	4.9	2.7	8.2	0.6	35.2		2.2	1.1	50.0	46.7
	40	33.0	11	7.3	3.7	3.6	8.0	0.7	38.7		1.8	0.9	51.3	49.4

*Experiment 1*—March 10, 1932. Cat. Blood non-protein nitrogen, 115; blood adenine nucleotide, 4.7 mg. per cent. Mg ion added to Sample 30-A.

*Experiment 2*—April 1, 1932. Cat. Blood non-protein nitrogen, 200; blood adenine nucleotide, 6.0 mg. per cent.

*Experiment 3*—April 18, 1932. Cat. Blood non-protein nitrogen, 118; blood adenine nucleotide, 5.0 mg. per cent.

*Preparation of Animals*—Experimental hyperthyroidism was induced in cats and rabbits by the intramuscular injection of solutions of crystalline synthetic thyroxine, kindly furnished by Hoffmann-La Roche, Inc. In the early experiments a commercially prepared solution containing 1 mg. of thyroxine per cc. was

used; later a solution made up just prior to use, containing 4 mg. per cc., was employed. Injections were carried out on alternate days and were continued until loss of weight, tachycardia, and obvious myasthenia indicated that the animals were thoroughly thyroxinized. Cats were found to be much less susceptible to thyroxine than rabbits. Doses given will be found in Table II.

TABLE IV  
*Carbohydrate Changes in Cat Gastrocnemii with Varying Amounts of Thyroxine*

Experiment No.	Total thyroxine	Autolysis	Lactic acid	Glycogen as lactic acid	Remarks
	mg.	min.	m.-eq.	m.-eq.	
1	2	0	12.3	95	1 injection
		10	21.2	19	
		20	38.4	12	
		40	43.4	8	
2	4	0	20.4	128	1 "
		10	28.7	45	
		20	50.9	18	
		40	49.4	15	
3	8	0	21.0	78	2 injections on alternate days
		10	30.7	24	
		20	35.3	7	
		40	44.9	7	
4	12	0	13.0	129	3 injections on alternate days
		10	26.0	40	
		20	33.6	35	
		40	39.2	20	
5	12	0	5.6	101	" "
		10	20.9	17	
		20	28.3		
		40	30.1	14	

In other experiments cats were adrenalectomized by Dr. George A. Harrop, Jr., and Dr. Albert Weinstein. The operation was performed in two stages, 5 days elapsing between the removal of the first and second glands. The animals were then allowed to develop adrenal insufficiency and were used only when myasthenia was severe. The gastrocnemii were removed and analyzed as described. Non-protein nitrogen and adenine nucleotide were determined in blood taken from the heart.

*Results*—Results are recorded in Tables I to VII. In each group of animals the individual variations were small. For this reason only one experiment typical of each group has been plotted

TABLE V  
*Autolyzing Cardiac Muscle of Normal Animals*

All results are expressed as milli-equivalents, either of P or of potential lactic acid.

Experiment No.	Autolysis <i>min.</i>	Lactic acid	Glycogen as lactic acid	Isolated Embden ester fraction					Ortho inorganic P	Phosphocreatine P	Adenylic pyrophosphate		Total acid-soluble P	
				Hexose as lactic acid	Calculated ester P	Free nucleotide P	Total organic P	Extra organic P			Pyrophosphoric P	Adenylic acid P	Determined total P	Calculated total P
1	0	3.7		8.4	4.2		7.0		4.8	1.1	14.8	7.4	34.7	35.1
	5	3.9	61	9.1	4.6		8.3		15.8	0.0	3.7	1.9	32.7	29.7
	10	9.3	37	8.7	4.4		9.3		17.4		1.7	0.9	33.7	29.3
	20	14.6	21	10.9	5.5		10.6		15.4		1.9	1.0	34.4	28.9
	40	18.1	21	12.0	6.0		12.0		14.9		2.5	1.3	35.2	30.7
2	0	3.1	107	10.6	5.3		7.2		8.7	2.0	7.9	4.0	33.4	29.8
	5	5.3	88	10.7	5.4		9.7		14.6	0.0	2.2	1.1	31.5	27.6
	10	9.8	69	15.8	7.9		11.6		12.8		1.3	0.7	31.2	28.4
	20	10.4	41	15.2	7.6		13.2		10.0		3.1	1.6	29.2	27.9
	40	13.1							10.1		3.4	1.7	31.9	
3	0	2.8	120	10.6	5.3	1.7	7.6	0.6	6.9	1.7	9.9	5.0	32.3	31.1
	5	3.7	98	9.9	5.0	2.2	7.4	0.2	9.6	0.9	8.1	4.1	33.5	30.1
	10	5.3	62	9.6	4.8	2.5	9.0	1.7	12.2		6.7	3.4	34.9	31.3
	20	12.2	57	12.2	6.1	2.4	10.5	2.0	11.6		5.9	3.0	33.2	31.0
	40	17.2	32	14.7	7.4	2.0	11.4	2.0	13.8		5.6	2.8	38.3	33.6
4	0	3.4	112	11.4	5.7		8.7		7.4	2.1	8.3	4.2	34.6	30.7
	5	5.0	93	13.8	6.9		12.3		12.0	0.6	3.4	1.7	35.2	30.0
	10	5.3	83	17.5	8.8		14.9		10.6		3.1	1.6	34.7	30.2
	25	6.5	60	16.8	8.4		14.6		7.8		1.5	0.8	33.4	24.7

*Experiment 1*—November 11, 1931. Two cat hearts.

*Experiment 2*—November 18, 1931. Three cat hearts.

*Experiment 3*—February 5, 1932. Three cat hearts.

*Experiment 4*—February 12, 1932. Three rabbit hearts.

in Figs. 1 and 2. To make results directly and easily comparable concentrations of all compounds containing P (except Embden ester) are expressed in terms of milli-equivalents of the element

P.<sup>2</sup> Glycogen, Embden ester, and lactic acid are all expressed in terms of milli-equivalents of lactic acid.<sup>3</sup> In the Embden ester 1 molecule of hexose (2 equivalents of lactic acid) is supposedly esterified with 1 equivalent of P. In Table VII results have been expressed in terms of mg. per cent of the element P, gm. per cent

TABLE VI  
*Autolyzing Cardiac Muscle of Thyroxinized Animals*

All results are expressed as milli-equivalents, either of P or of potential lactic acid.

Experiment No.	Autolysis <i>min.</i>	Lactic acid	Glycogen as lactic acid	Isolated Embden ester fraction					Orthoinorganic P	Phosphocreatine P	Adenylic pyrophosphate		Total acid-soluble P	
				Hexose as lactic acid	Calculated ester P	Free nucleotide P	Total organic P	Extra organic P			Pyrophospho-ganle P	Adenylic acid P	Determined total P	Calculated total P
1	0	3.5	43	8.2	4.1		8.6		7.6	1.8	7.5	3.8	32.5	29.3
	5	4.3	34	7.8	3.9		8.9		11.4	0.7	4.3	2.2		27.5
	10	7.4	27	8.5	4.3		10.7		15.6		1.1	0.6	36.2	28.0
	20	11.6	14	9.0	4.5		11.5		14.2		5.0	2.5	37.2	33.2
	40	18.0	10	9.0	4.5		10.8		15.2		5.0	2.5	36.4	33.5
2	0	4.1	58	8.2	4.1	3.2	6.7	-0.6	8.9	0.4	10.3	5.2	33.4	31.5
	5	5.8	36	8.5	4.3	3.6	7.0	-0.9	11.8	0.1	5.6	2.8	32.5	27.3
	10	6.0	17	7.7	3.9	4.6	7.5	-1.0	13.8		4.0	2.0	30.6	27.3
	20	10.7	10	9.3	4.7	3.3	8.1	0.1	12.6		5.5	2.8	31.6	29.0
	40	13.6	11	9.1	4.6	2.4	7.4	0.4	14.4		2.8	1.4	29.6	26.0
3	0	2.7	37	7.9	4.0	2.0	5.9	-0.1	8.7	1.0	9.9	5.0	34.4	30.5
	10	6.3	11	7.4	3.7	3.1	7.0	0.2	16.2		2.3	1.2	31.2	26.7
	20	8.9	10	6.7	3.4	2.4	7.8	2.0	16.1		1.8	0.9	29.8	26.6
	40	10.7	8	5.0	2.5	2.0	6.0	1.5	16.5		1.0	0.5	28.1	24.0

*Experiment 1*—February 20, 1932. Three cat hearts.

*Experiment 2*—May 16, 1932. Three cat hearts.

*Experiment 3*—June 13, 1932. Two cat hearts.

of glycogen as glucose, mg. per cent of Embden ester as hexose, and mg. per cent of lactic acid as such in order that these may be readily compared with values reported in the literature.

<sup>2</sup> To convert these figures into mg. per cent of P, multiply by 3.1.

<sup>3</sup> To convert into mg. per cent of hexose or glycogen, multiply by 9.

TABLE V.  
Comparative Composition of Cardiac and Gastrocnemius Muscles under Varying Conditions

Species	Muscle	Condition	Total soluble P		Inorganic P		Phospho-creatine P		Adenylic pyro-phosphate P		Ester P		Free adenylic acid P		Lactic acid	Glycogen as glucose	Average values for
			mg. per cent	per cent of total	mg. per cent	per cent of total	mg. per cent	per cent of total	mg. per cent	per cent of total	mg. per cent	per cent of total	mg. per cent	per cent total	mg. per cent	gm. per cent	
Cat. ....	Heart	Normal	103	21.7	21	5.3	5	48.4	47	14.3	14	5.3*	5	29	1.02	8 hearts (3 experiments)	
" .....	"	Thyroxinized	103	25.7	25	3.4	3	42.8	42	12.4	12	7.5	7	28	0.42	" "	
" .....	Gastrocnemius	Normal	175	51	29	33	19	65	37	65	10	3	2	90	0.88	3 animals	
" .....	"	Thyroxinized	154	42	27	31	20	62	40	62	8	2	2	50	0.42	3 "	
Rabbit. ....	Heart	Normal	107	23	21	6.5	6	39	36	39	17			28	1.01	3 hearts (1 experiment)	
" .....	Gastrocnemius	"	162	33	20	34	21	66	41	66	13			94		1 animal	
Cat. ....	"	Adrenalectomized	152	28	18	30	20	96	63	11	7	1		35	0.63	3 animals in insufficiency	

\* A single determination.

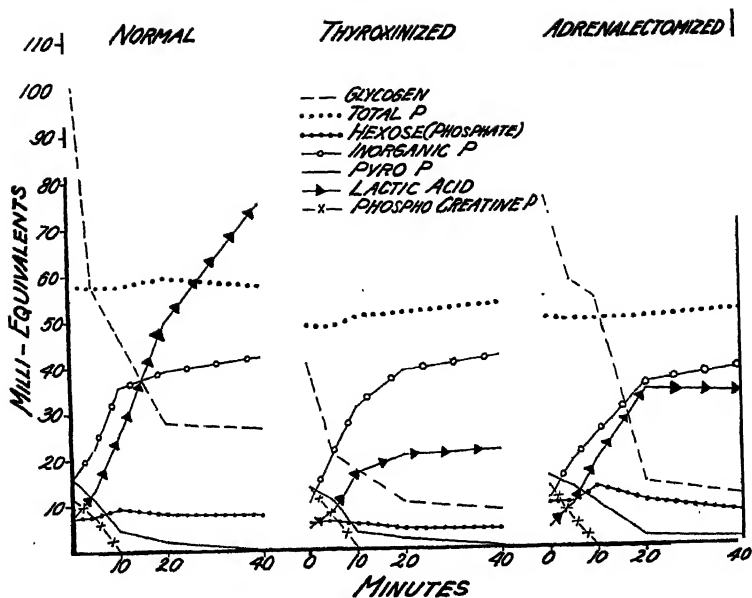


FIG. 1. Autolyzing cat gastrocnemius muscle

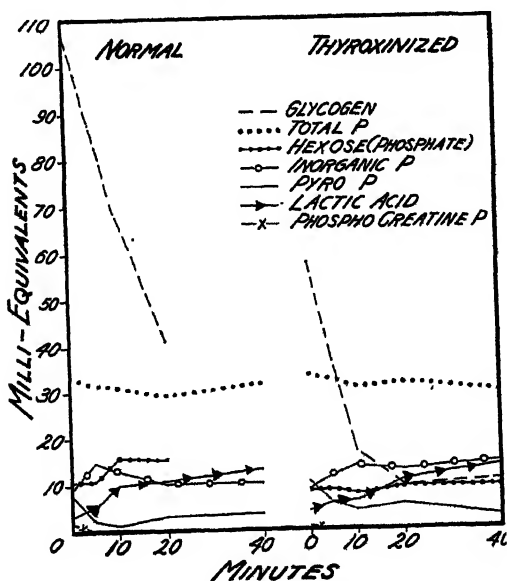


FIG. 2. Autolyzing cat heart muscle



## DISCUSSION

*Total P*—Most investigators are agreed that the total P extracted from a muscle by trichloroacetic acid, *i.e.* the acid-soluble P, remains constant during autolysis. This we have found to be the case in both gastrocnemius and heart muscle, within the limits of experimental error.

The initial total P content of cat and rabbit gastrocnemii was approximately the same; the total P of the heart averaged 60 per cent of that of the gastrocnemius. In both gastrocnemius and heart, under all the conditions studied (except in two of the adrenalectomized animals) the determined total P was satisfactorily accounted for in terms of the known P compounds which were studied; *i.e.*, orthoionorganic phosphate, phosphocreatine, adenylic pyrophosphate, and Embden ester fraction (Embden ester plus free adenylic acid). In gastrocnemius muscle about 97 per cent of the P was thus accounted for; in heart, 92 per cent. It cannot be concluded, however, that there are no unidentified P compounds in the acid filtrate, because there are certain assumptions inherent in some of the methods employed and also because other determinations must be regarded as giving minimum values (*i.e.*, only 93 per cent of the Embden ester is actually determined). This approximate agreement between calculated and determined total P, therefore, does not preclude the presence in the acid muscle filtrate of unidentified compounds of P; it indicates rather that, if present, these must exist at any one time in small quantities.

The total acid-soluble P of the heart (as well as the lactic acid production) was identical in normal and thyroxinized animals; in the gastrocnemii of the thyroxinized animals, however, the total P (and lactic acid production) was consistently and appreciably lower than normal, the level apparently bearing some relation to the degree of thyroxinization. This difference may be even greater than is apparent considering the fact that the muscles of the thyroxinized animals were very lean. No significant difference was found in moisture content. The total P (and lactic acid production) in the gastrocnemii of cats in insufficiency following adrenalectomy was also definitely low.

*Orthoionorganic P*—The orthoionorganic P of the gastrocnemii of normal, of thyroxinized, and of adrenalectomized animals increased rapidly in all cases during the first 10 minutes of autolysis, at the

expense of the labile phosphocreatine and the pyrophosphoric acid associated with adenylic pyrophosphate. Consequently the exact level of the initial ortho-inorganic phosphate must have depended on the degree to which autolysis had been prevented before fixation of the muscle, and must be regarded as a maximum value if interpreted as a measure of the *in vivo* inorganic phosphate P. The initial inorganic P was the same in normal and thyroxinized animals but was lower than normal in the adrenalectomized animals. The rate at which the inorganic phosphate was set free during autolysis was identical in muscles from normal, thyroxinized, and adrenalectomized animals. These results afford no direct proof as to whether the inorganic P set free from the decomposition of phosphocreatine and adenylic pyrophosphate accumulates directly without taking part in subsequent reactions or whether it is either partially or completely used momentarily to esterify a hexose and thus to form the hypothetical, evanescent hexose-, mono-, or diphosphate.

The initial value for ortho-inorganic phosphate was lower in heart than in gastrocnemius muscle, but represented approximately the same percentage of the total P in each tissue. In the course of autolysis, however, certain striking differences developed. In the heart a maximum was reached in 5 to 10 minutes, after which the inorganic P dropped appreciably and consistently, to rise again a little later. Moreover, this loss of ortho-inorganic P was quantitatively reflected by a rise in the pyrophosphate and ester fractions. Synthesis of pyrophosphate and ester was not similarly demonstrable, but undoubtedly occurred, in the autolyzing gastrocnemius.

The changes produced by autolysis in the heart muscle were relatively small as compared with gastrocnemius; consequently relationships were more difficult to establish with certainty. This very slowness with which autolysis proceeded, however, offered the advantage to the analyst that the initial values undoubtedly represented *in vivo* conditions more accurately.

*Phosphocreatine*—Just as the initial inorganic P must be regarded as a maximum value, the phosphocreatine P must be regarded as a minimum value.<sup>4</sup> The *average* figures for the gastroc-

<sup>4</sup> In fact, our values are about half of those found by Fiske and Subbarow (15). This means that our inorganic P is correspondingly higher.

nemii from four thyroxinized animals and three adrenalectomized animals did not vary from normal, although it may be significant that in both groups the lowest phosphocreatine values were found in muscles from the animals which were most severely myasthenic. A larger series of experiments in which the direct method of determination is adapted to small muscles is necessary to establish this point. In all cases complete decomposition of phosphocreatine had occurred after 10 minutes autolysis.

That heart muscle contains less phosphocreatine than does skeletal muscle has already been recognized; this fact has been correlated with the nature of cardiac activity (a single contraction followed by rest) in contrast to that of skeletal muscle. The amount of phosphocreatine found in the hearts of cats and rabbits was small. The gastrocnemius muscle, as analyzed, contained 6 times as much as did heart muscle; in the living animal the difference was undoubtedly even greater.

*Embden Ester*—Although several hexosephosphates have been isolated from muscle chemically treated in such a way as to alter the normal course of metabolism, only one, a hexosemonophosphate, the Embden ester, has been isolated from normal untreated muscle. The occurrence of other hexosephosphates in untreated muscle is hypothetical. The function of the Embden ester has not been satisfactorily established.

In contrast to phosphocreatine, Embden ester occurs in heart muscle in the same concentration as in gastrocnemius (or sometimes even greater) and, relative to the total P, in much greater quantities. This fact is significant in view of the vast difference in the quantity of lactic acid produced by the two tissues, and will be referred to again later.

In the normal gastrocnemius the Embden ester remained remarkably constant throughout the 40 minute autolysis period except for a small rise after 5 to 10 minutes. Nevertheless, lactic acid was formed at a rapid, uniform rate. We see no evidence in these experiments to support the idea that this ester is the precursor of the major part of the lactic acid formed on autolysis. The rise in Embden ester referred to was less apparent in the gastrocnemii of adrenalectomized animals and was not observed in the gastrocnemii of thyroxinized animals. Differences, however, were too small to emphasize and may have been influenced

by the fact that in the thyroxinized animals the glycogen stores were very low. Nevertheless, there was always sufficient glycogen present to permit the formation of Embden ester.

In the hearts of normal animals, too, a rise in Embden ester was observed after a few minutes of autolysis. Similarly, this increase was not observed in the hearts of thyroxinized animals. Here, too, glycogen was lower in the tissue from the thyroxinized animal than in that from the normal animal.

*Free Adenylic Acid*—In normal gastrocnemii there are only traces of free adenylic acid; in normal hearts there are significant quantities. This observation became apparent in connection with the study of the Embden ester. Cori and Cori (16) recognized the fact that, if present, free adenylic acid would be included in the barium precipitate by means of which the ester was isolated, a complication which would not affect the reducing properties but would increase the organic P of this fraction, and thus disturb the theoretical ratio between hexose and P. In gastrocnemius muscle, a theoretical ratio was obtained in the initial sample (fixed immediately), but as autolysis proceeded, an increasing divergence from the theoretical was observed. Thus it became necessary to determine directly the free adenylic acid to ascertain whether or not the extra P in the Embden ester fraction was indeed attributable to adenylic acid (and its deaminization product, inosinic acid). More data are necessary to establish this point definitely. In the muscles of normal and of adrenalectomized animals there is no evidence of the accumulation on autolysis of an unidentified form of P in this fraction. In the tissues of thyroxinized animals such a compound may exist.

*Adenylic Pyrophosphate*—In these experiments it is shown that in both gastrocnemius and cardiac muscle pyrophosphoric acid is rapidly split off from adenylic pyrophosphate, the initial rate of decomposition being only slightly slower than that of phosphocreatine. The gastrocnemii of thyroxinized cats showed initial values similar to the normal; those of adrenalectomized cats were higher than normal.<sup>5</sup>

<sup>5</sup> Curiously, the calculated total P in the gastrocnemii of adrenalectomized cats was higher than the observed value. Even if it is assumed that the whole burden of error was borne by the pyrophosphate determination, the corrected pyrophosphate values remain high.

The average absolute value for adenylic pyrophosphate in cat heart is 70 per cent of that in cat gastrocnemius; relatively, however, it represents a somewhat larger percentage of the total P.

It has been shown by one of us (24) that pure adenylic acid prepared from beef heart when brought to pH 7 and added to autolyzing muscle could be quantitatively accounted for as inosinic acid, without any dephosphorylation. This is demonstrated again (Table I, Experiment 4) by the fact that there is no increase in the inorganic phosphate produced when pure adenylic acid brought to pH 7 is added to autolyzing muscle. This, of course, is not conclusive proof that some of the naturally occurring nucleotide may not be dephosphorylated.

*Lactic Acid and Glycogen.* (a) *In Normal Gastrocnemii*—In the autolyzing normal muscle lactic acid was formed at an approximately constant rate throughout the 40 minute autolysis period; i.e., the production of lactic acid approximated a linear function of time. During the first 10 minutes of autolysis in cat muscle the lactic acid and inorganic P curves ran parallel; in rabbit muscle, in a long series of experiments not reported in detail, they were almost identical. These facts suggest anew, but do not prove, the existence of a common precursor for lactic acid and inorganic phosphate. It is usually assumed that an unidentified labile hexosemonophosphate is the active precursor. However, if both molecules (lactic acid and inorganic phosphate) had their origin exclusively in a common source, that source must have been a hexosediphosphate. Unlikely though it may seem, the possibility must be recognized that part of the inorganic phosphate may have originated directly from the decomposition of phosphocreatine and adenylic pyrophosphate without intermediary esterification of hexose. The appearance of equimolecular amounts of lactic acid and inorganic phosphate has been observed previously by other investigators when the rate of production of these ions was followed in muscle to which glycogen or some other substance was added. With the discovery of the Embden ester as the only demonstrable hexosephosphate in untreated muscle, the idea that a hexosediphosphate might serve as the immediate precursor of lactic acid has been discarded. Our survey of the P compounds in autolyzing muscle precludes the existence of any large amount of hexosediphosphate at any one time under the conditions of our experiments. If formed, it must be rapidly decomposed.

After the first 10 minutes of autolysis, formation of inorganic phosphate does not keep pace with production of lactic acid, since its sources, pyrophosphate and phosphocreatine, have been depleted by this time. Nevertheless, lactic acid continues to increase at a constant rate. This must mean that the inorganic phosphate can be utilized repeatedly for esterification if the same mechanisms of lactic acid production obtain throughout the autolysis.

(b) *In Gastrocnemii of Thyroxinized Animals*—The lactic acid production during autolysis of gastrocnemii from thyroxinized animals afforded a striking variation from the normal. In severely thyroxinized animals it differed from the normal in three respects: (1) the initial value was lower (50 mg. per cent instead of 90), (2) the rate of lactic acid formation was slower, and (3) lactic acid formation ceased sooner than in the normal. In fact, the quantity of lactic acid produced in 40 minutes by a muscle from a severely thyroxinized animal was only 20 to 30 per cent of that produced by a normal muscle. This failure of lactic acid production was not associated with any striking quantitative abnormalities in phosphocreatine, adenylic pyrophosphate, or Embden ester. It was, rather, associated with distinctly low glycogen content, and on first thought it might be supposed to be the direct result of the low glycogen content. That this is not the case, however, is indicated by Experiments 1 to 5 in Table IV. It will be seen that in animals which received only small amounts of thyroxine, lactic acid formation was somewhat impaired though the muscle glycogen was not perceptibly lowered. Surprisingly small, single doses of thyroxine caused a variation from normal in the lactic acid production, measured 48 hours after injection. The conclusion seems justified that impaired lactic acid formation was not caused directly by failure of an adequate supply of the ultimate precursor, glycogen. In the normal gastrocnemii lactic acid production continued at a uniform rate even after the maximum amount of inorganic phosphate was reached, thus indicating a continued resynthesis and breakdown of active intermediary. In the gastrocnemii of thyroxinized animals this did not occur; never did the lactic acid curve rise above the inorganic P curve (Fig. 1). We interpret this fact as indicating a specific failure of the mechanism for esterification of hexose to form the active intermediary (hexosediphosphate?).

The fact has already been mentioned that the exact level of normal, resting muscle lactic acid observed will vary with the degree to which autolytic changes are prevented before the muscle is fixed; a certain variation among normal values is to be expected. In those animals which received relatively small amounts of thyroxine (2 to 12 mg.), however, the initial lactic acid content was distinctly higher than could be accounted for by incidental variations in technique. The severity of the symptoms produced by thyroxine cannot be related quantitatively with the amounts given since there were wide variations in the reactions to thyroxine of different individuals of the same species (cat).<sup>6</sup> Nevertheless, our experience with partially thyroxinized cats confirms that of Andrus (25) working with rabbits which had received enough thyroxine to induce tachycardia and loss in weight, inasmuch as both groups showed an increased initial lactic acid content. With severely thyroxinized cats and rabbits, however, we have found consistently lower initial muscle lactic acid values than the average normal.

It might be urged that the limited autolytic formation of lactic acid by muscles taken from thyroxinized and adrenalectomized animals was associated with an altered acid-base balance, since Kondo (26) as early as 1912 found that the addition of acid inhibited the autolytic formation of lactic acid, whereas the addition of bicarbonate stimulated its formation. Undoubtedly even small changes in the pH at which autolysis takes place influence the quantity of lactic acid produced. We have carried out numerous autolyses of gastrocnemii from thyroxinized animals in 1 and 2 per cent  $\text{NaHCO}_3$  solution in which the relative quantities of muscle and bicarbonate were varied, in each case running a control autolysis in physiological saline. Sometimes less lactic acid was formed in the bicarbonate autolysate than in the control, sometimes more; but never was the increment in lactic acid sufficient to suggest that any relative lack of buffer capacity in the muscles from thyroxinized animals might be the

<sup>6</sup> One animal, Experiment 4, Table II, had lost only 13 per cent of its body weight after a total quantity of 53 mg. of thyroxine. On analysis, the picture was almost normal; whereas another, Experiment 3, Table II, after 6 mg. of thyroxine presented the most extreme picture of experimental hyperthyroidism.

predominating cause for their impaired ability to produce lactic acid, when autolyzing in physiological saline, as compared with muscle from normal animals.

There are additional reasons why it seems improbable that altered pH alone is responsible. The pH of a muscle poisoned with iodoacetic acid, in which no lactic acid is formed, is shifted in the direction of alkalinity (1). Furthermore, it was observed by Hoet and Marks (27) that the muscles of rabbits dying after prolonged thyroid feeding developed rapidly a rigor mortis which was not accompanied by the accumulation of lactic acid or by increased hydrogen ion concentration.

In all of the severely thyroxinized animals the glycogen content of the gastrocnemii was distinctly low, a fact which has been observed repeatedly by other investigators (12) working under different conditions. From the data in Table II one gets the impression that the muscle glycogen varies inversely with the degree of thyroxinization. As to the cause of the low glycogen one can only speculate, but the idea suggests itself that the thyroxinized animal may have lost to some extent its ability to resynthesize glycogen from lactic acid. This possibility will be investigated. The lactic acid production measured in these experiments is that produced by autolysis, and must not be confused with that produced by exercise. We have found, as have others, that the blood lactic acid after exercise was higher in patients suffering from hyperthyroidism or Addison's disease than in the normal. Theoretically this might be due either to an impaired ability to metabolize the lactic acid formed or to an overproduction of lactic acid by the contracting muscle.

(c) *In Gastrocnemii of Adrenalectomized Animals*—Cats suffering from adrenal insufficiency following bilateral adrenalectomy presented much the same picture of profound myasthenia as that shown by severely thyroxinized animals. Similarly, on analysis of the autolyzing gastrocnemius muscle, the striking deviation from normal was the rate and extent of lactic acid production. Lactic acid started at a lower level than normal, rose more slowly, and its production terminated sooner than in the normal muscle. Glycogen, also, was characteristically low. Never was the picture so extreme as that observed in the gastrocnemii of the most severely thyroxinized animals. It seems probable, therefore,



that in this condition also lactic acid production failed for the same reason; *i.e.*, inability of the muscle to synthesize the active intermediary.

The observation that skeletal muscles from thyroxinized and from adrenalectomized animals show an abnormality in lactic acid formation on autolysis is the more interesting in the light of the modern theory of the physiological rôle played by lactic acid. It is now believed that the conversion of glycogen into lactic acid is a reaction by means of which energy is furnished to the muscle, which permits of the resynthesis of phosphocreatine, the compound whose decomposition provides the immediate source of energy for contraction. If the continuous resynthesis of phosphocreatine were interfered with for any reason at all, such a handicapped muscle might logically be expected to have a relatively limited capacity for work and to excrete some creatine in lieu of reconvertng all of it into phosphocreatine. It is well known (28) that patients suffering from hyperthyroidism or from Addison's disease characteristically show creatinuria.

If it transpires, as is suggested by these observations, that the low glycogen content of muscle both in hyperthyroidism and in adrenal insufficiency is indeed attributable to impaired ability of the organism to convert lactic acid into glycogen, it appears that this inability to store glycogen may not be a manifestation of the specific influence of thyroxine or of adrenal insufficiency, but rather of an underlying condition common to both.

(d) *In Hearts of Normal and of Thyroxinized Animals*—It has already been stated that the rate of lactic acid production by the normal heart muscle is very much slower than that by the normal gastrocnemius. After 40 minutes the concentration of lactic acid in the former was about one-fifth of that in the latter. In contrast to the gastrocnemius, in which even small doses of thyroxine exerted an appreciable effect and large doses a profound effect on the autolytic production of lactic acid, no such apparent difference was observed in the hearts of severely thyroxinized animals. The curves (Fig. 2) representing lactic acid formation in the hearts from normal and from severely thyroxinized animals were essentially indistinguishable.

The previously recorded estimations of the lactic acid content of normal heart muscle agree more closely than do those of skeletal

muscle. This perhaps may be ascribed to the slow rate of lactic acid formation on autolysis in heart muscle as compared with gastrocnemius. Katz and Long (29) found the stimulation maximum for lactic acid much lower in heart than in skeletal muscle, a fact which they could not attribute to lack of glycogen. These authors urged in explanation, among other possibilities, the greater susceptibility of the heart to hydrogen ions. It has been demonstrated by Andrus (30) that such functions as the rate of beat of a surviving heart could be correlated within certain limits with the pH of the perfusing fluid, although for a given pH different buffer mixtures were more or less effective in altering the rate.

Considerable interest has centered about the effect of thyroxine on the glycogen content of heart muscle. A review of the literature reveals great discrepancy in the values reported for normal hearts of a given species; some investigators have found it to be only a small fraction of that found in skeletal muscle, while others have found as much or even more in heart muscle than in skeletal (22, 31-33). Apparently the technique of removal and subsequent handling of heart, as well as of skeletal muscle, and extreme care to avoid loss of glycogen in the various procedures involved in its determination are vitally important in securing glycogen values which represent *in vivo* conditions. We have found the glycogen of heart and gastrocnemius to be approximately the same (sometimes a little higher in heart); *i.e.*, about 1 gm. per cent.

In view of the uncertainty of the normal glycogen content of cardiac muscle it is small wonder that there is disagreement concerning the effect of thyroxine on it. Most investigators, however, have found a definite decrease in the glycogen content of the hearts of animals fed desiccated thyroid or injected with solutions of thyroxine (27, 34, 35).

In this series of experiments the cardiac glycogen was found to be low in all animals which had received enough thyroxine to cause loss of weight. The effect of small doses was not investigated in the case of heart muscle. The hearts of severely thyroxinized animals contained enough glycogen, however, to rule out low glycogen content as the ultimate cause for the low lactic acid production observed.

*Summary of Facts Relating to Lactic Acid Formation*—The observations reported above indicate certain fundamental metabolic differences between cardiac and skeletal muscle. These may be summarized as follows: (1) Although heart and gastrocnemius muscles have approximately the same initial glycogen content, the rate of production of lactic acid on autolysis is vastly different in the two tissues, being much more rapid in gastrocnemius muscle. (2) In the early stages of autolysis (first 10 minutes) the rates of production of lactic acid and of inorganic phosphate in gastrocnemius muscle are not only rapid but equal. (3) In the corresponding period lactic acid production in heart muscle is small and, as compared with that in gastrocnemius, is almost negligible. Here the appearance of inorganic phosphate bears no obvious quantitative relation to formation of lactic acid. (4) Phosphocreatine content of heart muscle is only a small part of that of gastrocnemius. (5) Thyroxine has a marked effect on lactic acid production in gastrocnemius, but no demonstrable effect upon lactic acid formation in heart muscle. (6) Embden ester content of heart and gastrocnemius muscles is essentially the same (and is not much altered as a result of thyroxine) in contrast to the vast difference in rates of lactic acid production by the two tissues.

Hence there appear to exist two mechanisms for the production of lactic acid: (1) the decomposition of the Embden ester which, though quantitatively small, may provide a *constant* source of energy for the resynthesis of phosphocreatine, and (2) a decomposition of a hypothetical intermediary compound (hexosediphosphate?) which may afford a source for the *rapid* formation of lactic acid. It appears further that the formation of lactic acid in cardiac muscle during autolysis can be ascribed principally to the first of these two mechanisms. In skeletal muscle, on the other hand, while both reactions appear to operate, the rapidity and magnitude of the second far outdistance the first. Although the mechanism of lactic acid formation characteristic of cardiac muscle suffers little interference from the action of thyroxine, that operating in gastrocnemius is markedly interfered with as a result of thyroxine. These findings reaffirm the fundamental difference between cardiac and skeletal muscle as regards the formation of lactic acid and support the suggestion that in cardiac

muscle lactic acid is formed principally by the first of the reactions described above.

#### SUMMARY AND CONCLUSIONS

1. A survey has been made of the known P compounds occurring in the gastrocnemius and heart muscles of normal cats and rabbits, the same muscles of thyroxinized cats, and the gastrocnemii of cats in insufficiency following adrenalectomy.

2. The relation of all of the compounds of P to each other and to the process of glycolysis has been followed in these muscles over an autolysis period of 40 minutes.

3. The acid-soluble P of these muscles, both in the initial instance and throughout the period of autolysis, could be almost quantitatively accounted for in terms of ortho-inorganic phosphate, adenylic pyrophosphate, phosphocreatine, Embden ester, and free adenylic acid.

4. Unidentified compounds of P may exist in only small quantities.

5. The total acid-soluble P of heart muscle was found to be 60 per cent of that of gastrocnemius.

6. In the normal gastrocnemius decomposition of phosphocreatine and of adenylic pyrophosphate takes place rapidly and soon ceases, whereas lactic acid production continues at its initial rate throughout the experimental period.

7. There is no obvious relation between the Embden ester and the total amount of lactic acid formed by gastrocnemius muscle.

8. The formation of equimolecular quantities of lactic acid and inorganic phosphate suggests that the precursor of most of the lactic acid in gastrocnemius muscle is a hexosediphosphate.

9. In gastrocnemii from severely thyroxinized animals lactic acid formation is inhibited, as evidenced by lower initial values, slower rate of formation, and earlier cessation of production than in the normal.

10. The glycogen content of the gastrocnemii of thyroxinized animals is more or less decreased, depending on the degree of thyroxinization. Decreased glycogen is not the cause of decreased lactic acid production. The possibility is suggested that the thyroxinized as well as the adrenalectomized animal may have lost in part its capacity for synthesis of glycogen from lactic acid.

11. In thyroxinized animals the total acid-soluble P of the gastrocnemius was lower than normal, with no significant change in the relative distribution of P.

12. In adrenalectomized animals, also, there was definite impairment of the ability of the gastrocnemius to produce lactic acid autolytically. Here, too, the total P was low, and relatively the pyrophosphate was higher and the inorganic phosphate lower than normal.

13. The hypothesis is suggested that the myasthenia and creatinuria characteristic of hyperthyroidism and of Addison's disease may be associated with the inability of the muscles to produce lactic acid normally, a condition which might limit the resynthesis of phosphocreatine.

14. The hearts of thyroxinized cats contained the same amount of acid-soluble P as the normal, in contrast to the gastrocnemii.

15. Normal cat heart contained less phosphocreatine, more free adenylic acid, the same or more glycogen, and less lactic acid than the normal gastrocnemius.

16. The glycogen of the hearts of thyroxinized animals was lower than normal; otherwise the composition of the hearts of normal and of thyroxinized animals was similar in respect to the compounds studied, and the autolysis curves were indistinguishable.

17. Beyond its effect in reducing the glycogen content of the tissue, the chemical action of thyroxine upon cardiac muscle remains to be demonstrated.

18. The hypothesis is suggested that there are two different mechanisms for lactic acid production.

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# THE CUPRIC COMPLEXES OF GLYCINE AND OF ALANINE

By HENRY BORSOOK AND KENNETH V. THIMANN

(From the William G. Kerckhoff Laboratories of the Biological Sciences,  
California Institute of Technology, Pasadena)

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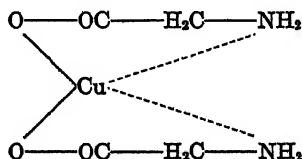
## I. INTRODUCTION

### (a) HISTORICAL

The following report is the first of a projected series of studies of the physical chemistry of the compounds of the heavy metals, particularly of copper and of iron, with substances of biological importance. These studies are invited by the accumulation in recent years of examples of the importance of the heavy metals in biological chemistry.

The copper compounds of glycine and of alanine were studied first, in the hope that the analysis of the factors affecting the formation of these relatively simple compounds would facilitate the elucidation of the more complex systems.

It has long been known that amino acids and cupric ion react to form stable complexes. Ley, in 1904 (1), suggested for the copper-glycine complex the formula



the dotted lines signifying secondary valencies. In support of this formula Ley and his collaborators later published spectrophotometric absorption data in the visible and ultra-violet regions for copper-glycine and for copper-alanine (2, 3). The chief evidence adduced by Ley for the accompanying formula was the similarity between the absorption spectra, in the visible region



of the copper complexes of the monoaminomonocarboxylic acids and those of the cupriammonium complexes.

Barker, in 1907 (4), reported the results of potentiometric and freezing point measurements in aqueous solutions of copper sulfate and glycine, from which the conclusion was drawn that 4 molecules of glycine were combined with 1 of copper, the combination being effected by the four secondary valencies of the copper with the nitrogen atoms of the amino acids. Most of the results published by Barker, without special interpretation, may be taken equally well to indicate that 3 instead of 4 molecules of glycine are combined with 1 of copper.

The interpretation of freezing point data here is complicated by the formation of aggregates between the amino acid molecules (5), which has only been discovered in the last few years, and which the earlier investigators failed to take into account. An additional uncertain correction must be introduced also for the activity coefficients of the electrolytes in solution.

These qualifications also weaken the evidence adduced by Pfeiffer (6) from freezing point measurements for the formation of complexes between neutral salts and amino acids. Direct evidence that these compounds may exist at least in the solid state was, however, obtained by Anslow and King (7), who crystallized a number of complexes of inorganic neutral salts and dicarboxylic amino acids.

In a series of interesting and suggestive studies Kober and Sugiura (8) and Kober and Haw (9) showed that cupric ion forms with monocarboxylic- $\alpha$ -amino acids compounds of the type  $\text{CuA}_n$ , where A refers to the amino acid molecule; and with glutamic acid, aspartic acid, isoserine, and all polypeptides, compounds of the type  $\text{CuA}$ . From the examination of a large number of compounds they deduced the rule that the region of absorption is shifted more and more toward the blue as the number of nitrogen atoms attached to the copper in a stable ring, either by secondary or primary valencies, increases from 2 to 4. Accordingly, in strong alkali monoamino acids and dipeptides give a blue color similar to that of cupriammonia, and an absorption maximum at 6300 Å.; tripeptides, a bluish violet with absorption maximum at about 5400 Å.; and tetrapeptides, a red color with absorption maximum at 4430 Å. The latter is the true biuret color, while the color given by proteins resembles that of the tripeptides.

Pfeiffer (6) has accepted the type of formula proposed by Ley for the copper compounds of glycine and alanine; *i.e.*, an internal complex with the primary valencies of the copper attached to the carboxyl groups. Ley and Pfeiffer, in their formulation of the copper compounds of the amino acids, have tacitly accepted the classical conception of the strength of the acid and basic radicals of the amino acids. Since the *Zwitter Ion* constitution of amino acids seems now firmly established (10), we have employed it throughout in designating the species of ionic form of the amino acid existing at any given hydrogen ion concentration.

The interesting point which the present study has revealed is the variation of the constitution of the copper-glycine or copper-alanine complexes present in any solution with the hydrogen ion concentration. This phenomenon seems, up to now, to have been almost entirely overlooked. Ley and Hegge (2) stated that copper-glycine can be obtained in two forms, in plate-like and needle-like crystals; but that no difference is detectable in solution. By conductivity and electrode potential measurements Barker found that the addition of glycine to solutions of zinc sulfate increased the hydrogen ion concentration of the solution; and from the similarity in behavior of the conductivities of glycine-zinc and glycine-copper sulfate solutions he concluded that the formation of a glycine-copper sulfate compound also increased the hydrogen ion concentration of the solution. This we have confirmed. Kober and Haw observed that the absorption of a given complex is somewhat dependent on the concentration of the hydroxyl ions. As far as the amino acid complexes are concerned they reported only that, "In the amino-acid blue complexes there is no visible change with weak alkali; but in strong alkali most of the copper is precipitated."

This oversight was due, it seems, to the practice of attempting to account for all the properties of solutions of copper compounds of glycine and of alanine by those of the crystals. This amounts in effect to considering only saturated solutions in which the copper and amino acid are in equivalent proportions. It is obvious that such compounds can be of only remote significance under physiological conditions where the concentration of nitrogenous substances is enormously in excess of that of the copper, which is present only in traces.

One of the few attempts to consider the equilibria in solution was made by Shibata and Matsuno (11). They noted that changes occurred in the absorption spectra of copper sulfate solutions with changes in concentration, but limited their explanations to the postulate of the formation of aquo complexes.

### (b) Absorption Spectra

The data presented below indicate that as the hydrogen ion concentration is varied from pH values of 0 to 13, at least four compounds of cupriglycine and cuprialanine are formed. In Figs. 1 and 3 the absorptions in the visible spectra of these four compounds are set out and compared with those of copper sulfate, of undissociated copper acetate (*i.e.* in alcoholic solution), and of cupriammonium sulfate.

The effect of varying the hydrogen ion concentration at constant relative concentration of amino acid to copper is shown in Figs. 2 and 4, which give only a few curves from the series of thirty-four obtained. As discussed in Section III, *a* it was impossible to interpret the changes in absorption in terms of less than four compounds. Furthermore, analysis of combined spectrophotometric and potentiometric data showed that in some solutions three copper-amino acid compounds were present at once. On this account it would be extremely difficult, if not impossible, to obtain from aqueous solutions the second acid copper-glycine or alanine complex in the pure crystalline state. Even if these compounds were prepared, on re-solution a rearrangement would occur, with the result that the solutions so formed would contain more than one complex. The same consideration, in lesser degree, applies to the other complexes. The pure neutral compound, if prepared and dissolved in water, would probably suffer the least change, though even here the absorption data of Ley and of Kober and Haw suggest that a detectable amount of the second acid complex is formed (*cf.* p. 683).

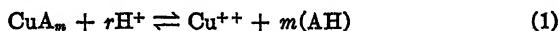
Since a solution of the crystals of any one copper-glycine or alanine complex would therefore at once become a mixture, the only way these compounds can be obtained alone in solution is by increasing the concentration of amino acid (with the pH kept constant within a suitable range) until no further change in the absorption of the solution occurs. By this method the absorption

curves of the pure neutral and basic complexes were obtained. The determination of the absorption curves of the two acid complexes—one, designated the first acid complex, predominating at pH 2.0, the other, the second acid complex, predominating at pH about 4.0—presented more difficulty, since their ranges of stability overlap. The curves deduced are therefore less certain than those of the neutral and basic complexes. The curves for the first acid complexes were obtained from dilute solutions of amino acid after the absorption due to the free cupric ion, whose concentration was determined potentiometrically, had been subtracted from the total absorption observed. The curves for the second acid complexes were obtained by a method of trial and error described below.

### (c) Potential Measurements

By means of copper electrode potential measurements we have also attempted to determine by the following method, due to Bodländer and Storbeck (12), the number of molecules of amino acid combined with 1 molecule of copper.

The general equation for the formation of any one of the complexes (where  $AH$  represents that form of the amino acid taking part in the reaction, and  $CuA_m$  the complex) is



In it the  $CuA_m$  bears a charge which varies with the complex under consideration and also contains  $(m - r)$  H atoms. The general treatment of the equilibria is facilitated (without being invalidated in any way) by omitting these from mass law equations. The mass law expression for the equilibrium is therefore

$$\frac{(Cu^{++}) \cdot (AH)^m}{(CuA_m) \cdot (H^+)^r} = K \quad (2)$$

where  $m$  is the number of molecules of amino acid in the complex and  $r$  the number of hydrogen ions set free in the formation of 1 molecule of complex.

Therefore, for two different amino acid and hydrogen ion concentrations we may write

$$\frac{(Cu^{++})_1 \cdot (AH)_1^m}{(CuA_m)_1 \cdot (H^+)_1^r} = \frac{(Cu^{++})_2 \cdot (AH)_2^m}{(CuA_m)_2 \cdot (H^+)_2^r} \quad (3)$$

which on conversion to logarithms and rearrangement becomes

$$m \log \frac{(\text{AH})_1}{(\text{AH})_2} = \log \frac{(\text{Cu}^{++})_2}{(\text{Cu}^{++})_1} + \log \frac{(\text{CuA}_m)_1}{(\text{CuA}_m)_2} + r(\text{pH}_2 - \text{pH}_1) \quad (4)$$

Since the potential difference between copper electrodes, in two solutions where all other ions except the cupric ions are at practically the same concentration, is

$$E = \frac{RT}{nF} \ln \frac{(\text{Cu}^{++})_1}{(\text{Cu}^{++})_2} \quad (5)$$

therefore, by being converted to base 10 and inserted in Equation 4 the latter becomes

$$m \log \frac{(\text{AH})_1}{(\text{AH})_2} = (E_1 - E_2) \frac{nF}{2.303 RT} + \log \frac{(\text{CuA}_m)_1}{(\text{CuA}_m)_2} + r(\text{pH}_2 - \text{pH}_1) \quad (6)$$

If practically the whole of the copper is in the complex form, the second term on the right-hand side disappears, and hence the value of  $m$ , *i.e.* the number of molecules of amino acid combined with 1 cupric ion in a given complex, can be obtained from the potential difference between copper electrodes, if the hydrogen ion concentrations are the same in the two solutions.

In those solutions in which an appreciable fraction of the copper is not in the complex form, a correction for this has to be applied. This was done either by assuming the copper electrode potentials to give absolute  $\text{Cu}^{++}$  concentrations, and deducting these from the total copper present, or else by algebraic analysis of the absorption curves, as described in Section III, *a*. Where possible, both methods were used, and the results checked each other fairly satisfactorily.

In acid solutions it was found that hydrogen ion concentrations great enough to suppress ionization of the  $\text{COOH}$  group of the amino acid also suppressed all complex formation (*cf.* Fig. 2, *A*). This means that only the *Zwitter Ion* form of glycine and alanine takes part in the complex formation with copper in the acid range. Consequently, the concentration of free amino acid employed in Equation 6 refers only to that in the *Zwitter Ion* form. This is therefore the difference between the total calculated concentration of *Zwitter Ion* form of amino acid at the pH of the solution and

that bound in the complex. Where the concentration of amino acid was greatly in excess of that of the copper, and the pH between 4.5 and 8.0, no error is incurred by taking as the concentration of free amino acid the total amount added initially.

For the determination of hydrogen ion concentrations in the presence of copper the glass electrode was used, as described in Section II, *b*.

When the value of  $m$  had been obtained from solutions in which the pH was the same, *i.e.* where only the amino acid concentrations were different, this value of  $m$  was then employed in other solutions for the determination of  $r$ . By using several determinations mean values for  $m$  and  $r$  were obtained, and from these the approximate equilibrium constants were computed.

In the case of the neutral compounds of glycine and of alanine, and of the second acid compound of alanine, another, independent, method of obtaining the value of  $r$  was also employed. This consisted in the measurement of the small change in hydrogen ion concentration of the amino acid solution, resulting from the addition of measured small amounts of copper sulfate solution. The method is described in Section III, *b*; the results are shown in Tables III, VII, and IX. In this method, when the amino acid concentration was greatly in excess of that of the copper, all of the copper could be considered to be in complex form; in more dilute solutions, the free copper, and hence by subtraction the amount of complex, was determined by means of the copper electrode.

## II. EXPERIMENTAL

### (a) Absorption Spectra

The absorption data were obtained with a König-Martens spectrophotometer. The cell employed was a T-piece 73.5 mm. long, with plate glass ends cemented on. This length of absorption solution permitted the use of low concentrations of copper (usually 0.002 M) and of correspondingly large variations in the relative excess of amino acid. In order to maintain the composition as uniform as possible with respect to  $\text{SO}_4$  ions, which facilitated the interpretation of the copper electrode potentials,  $\text{K}_2\text{SO}_4$  was added to all solutions to a final concentration of 0.1 M. Owing to the large error which traces of opalescence introduce,

all solutions were filtered into the absorption cell before measurements were made. To allow for absorption due to water and to reflection by the cell surfaces, blank determinations were made on distilled water similarly filtered into the cell.

### *(b) Hydrogen Ion Concentrations*

In the solutions marked \* in Tables I, II, and V, a quantity of  $\text{H}_2\text{SO}_4$  estimated to be equivalent to the hydrogen ions set free by the formation of the copper-amino acid complex was added to the solution of amino acid, together with potassium sulfate, and the acid or alkali necessary to bring the hydrogen ion concentration of the solution to the desired pH. The pH was then measured electrometrically with a Moloney hydrogen electrode (13). The value obtained was checked colorimetrically on the similar solution containing  $\text{CuSO}_4$  (instead of the excess acid) on which the spectrophotometric and copper electrode potential measurements were carried out. The pH values given for these solutions are uncertain to  $\pm 0.2$  pH units.

In the solutions marked † in Tables I, II, V, VI, and VIII, the hydrogen ion concentrations were measured in the final mixture containing copper. This was done with the glass electrode, the modified electrical arrangement described by Robertson (14) being used. With this set-up, potentials were determined by means of a high sensitivity galvanometer (Leeds and Northrup type 2500) and a type K Leeds and Northrup potentiometer, without the use of vacuum tubes. The electrodes were made from the special Corning glass employed by MacInnes and Dole (15). The electrode used was calibrated before each measurement with buffer solutions whose pH values bracketed that of the solution to be measured. The uncertainty of the values obtained was not greater than 0.02 pH unit.

### *(c) Copper Ion Concentrations*

Copper foil electrodes have been used in complex ion work by Riley (16). The use of foil or wire electrodes, however, was shown by Getman (17) to introduce variations in the potential according to the metallurgical treatment of the copper, and the electrodes here employed were therefore prepared according to the directions of Lewis and Lacey (18) and of Getman (17).

Spongy copper was obtained by electrolyzing a solution of twice recrystallized  $\text{CuSO}_4$  between platinum foil electrodes with a high current density, so that the copper was deposited in streamers of spongy metal which did not adhere to the cathode but sank to the bottom of the beaker. This was thoroughly washed in freshly boiled distilled water (redistilled through a block tin condenser) until quite free from  $\text{SO}_4$  and then set away in a bottle filled with freshly boiled redistilled water to preserve it from oxidation in the air. The copper electrodes consisted of platinum wire very thinly covered by a film of copper deposited out of a 0.01 M  $\text{CuSO}_4$  solution by means of the current from one dry cell for 30 seconds. These electrodes were then covered by spongy copper which had been washed several times with the solution in which the electrode was finally brought to equilibrium. Before being used on amino acid solutions the electrodes were checked by measuring the potential difference between known concentrations of copper sulfate in the two electrodes, usually 0.01 M against 0.001 M. The potential difference found at 25° for this concentration difference was 22 millivolts, the identical value obtained by Labendzinski (19) for this cell. When both  $\text{CuSO}_4$  solutions contained 0.1 M  $\text{K}_2\text{SO}_4$ , values of 29.5 to 30.1 millivolts were obtained. In the operation of this cell it was found that difference in hydrogen ion concentration produces a large liquid junction potential. Accordingly, when the cupric ion potential difference between solutions containing different concentrations of amino acid was desired, the pH values of the standard and the unknown solutions were made as nearly as possible the same. In spite of these precautions discrepancies sometimes occurred between the values for the concentration of free cupric ions, deduced from copper electrode potential, and those obtained from spectrophotometric data. It is possible that this was due to a solution of metallic ion by the acid in the presence of small amounts of dissolved oxygen. In most cases, however, the values found by potentiometric and spectrophotometric methods were in fairly good agreement.

The usual procedure in the determination of the cupric ion potential was as follows: The two electrode vessels, one containing the standard copper sulfate solution, the other the amino acid and consequently an unknown concentration of cupric ions, connected by an intermediate solution which was the same as the



standard, were set away in an air bath at 25°, with the stop-cocks closed. These were opened only while readings were being taken. Fresh liquid junctions were made for each reading by opening screw clamps on top of each electrode vessel. Readings were taken from time to time until the potential was observed not to drift more than 1 millivolt in 1 hour. The time elapsed was as a rule from 2 to 3 hours.

The employment of 0.1 M  $K_2SO_4$  in the standard and in the solutions containing amino acid eliminated potentials due to  $SO_4$  ion concentration difference, and maintained a nearly constant ionic strength in all solutions. It was hoped that this nearly constant concentration of strong electrolyte would minimize the effect of varying amino acid concentrations on the activity of the cupric ion, and, on the assumption of constant ionic strength, would justify the calculation directly from the potentials of cupric ion concentrations instead of cupric ion activities.

The copper sulfate used in the preparation of the spongy copper and in the solutions was twice recrystallized from a c.p. specimen, care being taken to obtain small crystals; and then powdered and dried at 120°. From this salt a stock 0.1 M solution was made which served for all subsequent dilutions.

The specimens of glycine and *DL*-alanine used were recrystallized several times from isoelectric solutions of commercial preparations. After drying, these gave the melting points cited in the literature, 235° for glycine and 295° for alanine.

### III. Cupric Salts of Glycine

#### (a) Absorption Spectra

Fig. 2 shows the variation in the absorption of solutions containing a constant concentration of  $CuSO_4$  (0.002 M) and of glycine (0.5 M) when the hydrogen ion concentration is changed from pH 0 to 12. Beginning with the absorption of free  $CuSO_4$  in extreme acidity, the absorption in the red rises, at first slowly, then more rapidly, and then falls again. The absorption in the neighborhood of 6250 Å. increases steadily and attains a constant value. From pH 5 to 8 the absorption remains constant. It was concluded, therefore—a conclusion corroborated by all subsequent analysis of the data—that all but a negligible quantity of the copper was bound in the form of only one complex, whose absorption curve was

that found in these solutions, from pH 5 to 8. This compound was designated as neutral copper-glycine.

In alkalinities higher than pH 8 a further increase in absorption was observed in the orange and red end of the spectrum. This was taken to indicate the formation of another compound, which was designated as basic copper-glycine. The extrapolation method by which the absorption curve was established is described below.

All attempts to interpret the absorption curves of the solutions more acid than pH 5 in terms of a neutral complex and of only one acid complex failed. The increasing and then diminishing absorption at the red end of the spectrum could be accounted for completely only by postulating a second acid complex occurring between the first acid and the neutral complexes. Similar results were obtained with alanine.

In Fig. 1 are set out the final absorption spectra of the four salts. The curve for the first acid copper-glycine (Curve 3) was derived from dilute acid solutions (pH 4.4 to 4.6) in which the free cupric ion was calculated from copper electrode potentials, amounting to 63 and 53 per cent of the total copper respectively; the absorption due to this amount of  $\text{Cu}^{++}$  was deducted from the total absorption. The accuracy of the curve so obtained was confirmed by the absorption of a solution containing 1 M glycine and 0.002 M copper at pH 2.05, where the large excess of glycine caused practically all of the copper to be combined in this form. The absorption of the first acid complex having been thus obtained, it was possible by means of simultaneous equations to solve, with good agreement, the absorption curves of other solutions in the acid range.

The general method for solving such curves is as follows:

Let  $x$  = fraction of total copper as cupric ion  
 $1 - x$  = " " " " in complex  
 $A_1$  = absorption of cupric ion at wave-length  $\lambda_1$   
 $B_1$  = " " copper in complex form at wave-length  $\lambda_1$   
 $\Delta_1$  = total absorption of solution at wave-length  $\lambda_1$   
 Then  $xA_1 + (1 - x)B_1 = \Delta_1$ . Similarly at another wave-length,  
 $xA_2 + (1 - x)B_2 = \Delta_2$ , etc.

Proceeding through the visible spectrum we obtain a value for  $x$ , from the solutions of pairs of simultaneous equations, which will

be constant throughout the spectrum if the absorption curves chosen, *i.e.* the values of  $A_1, A_2, A_3, B_1, B_2, B_3$ , etc., are correct. Values for  $A_1, A_2, A_3$ , etc., were obtained from a pure copper sulfate solution at high dilution (see Curve 1, Fig. 1). If a constant value of  $x$  is obtained for any given set of values for  $B_1, B_2, B_3$ , etc., the accuracy of these values for the absorption spectrum of the unknown compound is established.

The absorption spectrum for the first acid compound so obtained was then combined with that for  $\text{CuSO}_4$  to yield concordant

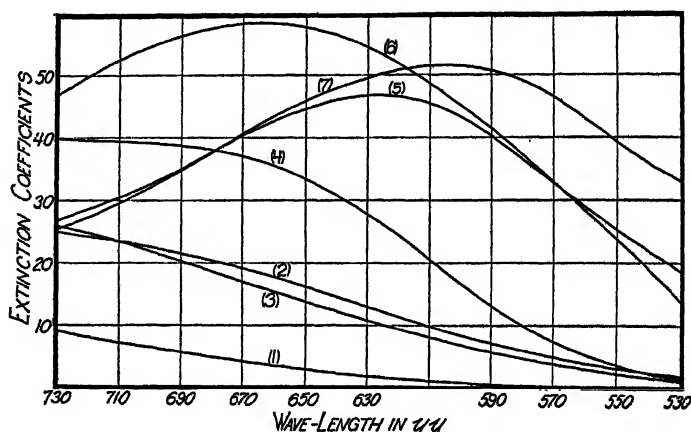


FIG. 1. Absorption curves of cupriglycine compounds. Extinction coefficients  $\log \frac{I_0}{I} \cdot \frac{1}{c} \cdot \frac{1}{L(\text{cm.})}$  at various wave-lengths in  $\mu\mu$ . Curve 1, copper sulfate; Curve 2, copper acetate in alcohol; Curve 3, first acid cupriglycine; Curve 4, second acid cupriglycine; Curve 5, neutral cupriglycine; Curve 6, basic cupriglycine; Curve 7, cupriammonium sulfate.

figures for the concentrations of each in solutions in which the glycine concentration varied from 1 M to 0.004 M, and the pH from 0.25 to 4.6. In spite of this concordance, it is still possible that this curve is too high. The absorption is so near to that of the  $\text{CuSO}_4$  that spectrophotometric data alone cannot lead to a very reliable curve for the first acid complex. If a lower absorption curve were taken, the simultaneous equations would yield different but, within the limits of experimental error, almost concordant values for the amounts of free and bound copper. Further, the curve

given is much higher than that for the corresponding copper-alanine, which is more firmly established. However, there is a good reason for this (see Section IV, b), and also the curve is supported by the agreement between potentiometric data for the amounts of free copper and those calculated from the absorption spectra. Its close resemblance to the curve of undissociated cupric acetate is also probably significant.

The absorption spectrum of the second acid compound was constructed arbitrarily, by trial and error, to account for the absorptions of the solutions between pH 3 and 6. This curve (Curve 4 in Fig. 1) was not obtained unmixed with those of other complexes in any solution. The absolute values are accordingly somewhat uncertain. However, combination of this curve with the others satisfactorily accounted for the absorptions of solutions ranging from 0.5 M glycine at pH 2.9 to 0.002 M glycine at pH 7.2. Thus the complex appears to exist in concentrated solutions, *i.e.* when glycine is greatly in excess of copper, around pH 4, and in neutral solutions when the concentrations of glycine and copper are nearly the same and both very dilute.

The neutral copper-glycine is the only form described in the literature. Its absorption curve (Curve 5 in Fig. 1) is based on the absorptions of solutions at pH 5 to 8 in which the concentrations of glycine varied from 0.05 to 1.0 M. Since practically all the copper in these solutions was bound in this form, the absorptions obtained were almost the same in spite of the 20-fold variation in concentration of glycine.

This absorption curve is higher than any previously quoted for this complex. Thus at 6250 Å., where we have found the extinction coefficient to be 47, Kober and Haw give 40, and Ley and Vanheiden (3) give 46. The explanation of these lower values lies in the fact that their solutions were prepared by dissolving crystals of copper-glycine in water, with a resulting total molal concentration of glycine only twice that of the copper. Some decomposition, therefore, occurred into the second acid form, and to a slighter extent into free  $\text{Cu}^{++}$  and glycine. The absorption would accordingly be less than that of a solution containing only neutral complex. However, the form of the curve given in both instances is the same as that given here.

The existence of a basic copper-glycine complex was indicated

from the change in absorption, clearly visible in the color of the solutions, beyond pH 8. The absorption of this basic form was obtained by extrapolating the values obtained in solutions of increasing alkalinity, containing 0.5 M glycine and 0.002 M copper sulfate. The quantities plotted were the excess absorption over that of the pure neutral complex, for each wave-length measured,

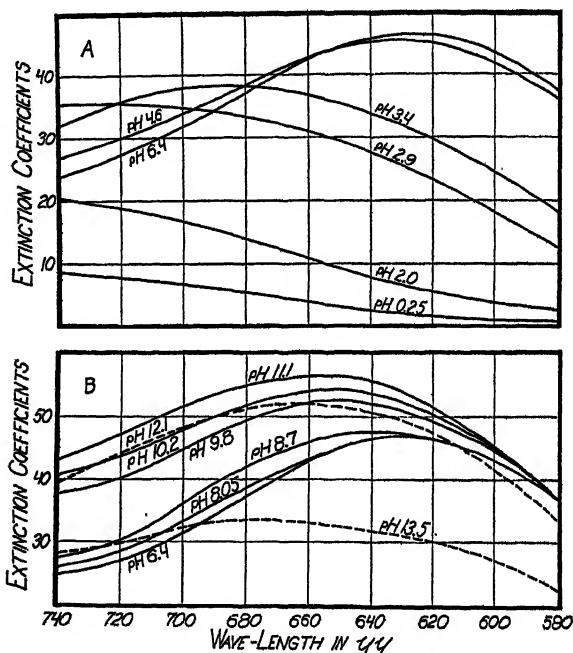


FIG. 2, A. Effect of changing H ion concentration on the absorption of cupriglycine solutions containing the same amounts of copper and of glycine; Cu = 0.002 M, glycine = 0.50 M; acid range.

FIG. 2, B. Same as Fig. 2, A; alkaline range. Solutions in which precipitation occurred are shown in broken lines (pH 12.1 and 13.5).

against pH. The curves so obtained were of the form of dissociation curves, and flattened rapidly towards pH 11.1. From each the absorption at one wave-length was taken, and the resulting extrapolated curve for the basic complex is Curve 6 in Fig. 1. By combining this curve with that for the neutral compound it was possible to solve with good agreement the absorption curves

of solutions in which the pH varied from 8.05 to 13.5 and the glycine concentration from 0.06 to 0.5 M.

The diminishing absorption beyond pH 11.1, shown in Fig. 2, *B*, is due in part at least to the precipitation of inorganic cupric hydroxide, since filtration of these solutions left a visible pale blue precipitate on the paper. Kjeldahl analysis of this precipitate showed it to be free of nitrogen. The curves in this pH region are therefore dotted in Fig. 2, *B*.

### (b) Potentiometric Data and Constitution

*First Acid Complex*—In Table I are collected the data from E.M.F. measurements, and the calculations of  $m$  and  $r$  for the first acid copper-glycine; *i.e.*, the number of molecules of glycine combined with 1 of copper, and the number of hydrogen ions set free in the formation of 1 molecule of complex. The data indicate that in this complex 2 molecules of glycine combine with 1 of copper, liberating 1 hydrogen ion.

In the calculation of the instability constant (Column 11) the concentrations of free *Zwitter Ion* glycine (Column 8) and not of total uncombined glycine were taken. This procedure is based on the observations shown in Fig. 2, and discussed in Section I, *c*, that complex formation progressively diminishes with suppression of ionization of the carboxyl group and disappears at high acidity; *i.e.*, only the *Zwitter Ion* glycine participates in this equilibrium.

The absorption spectra of the first two solutions in Table I were analyzed algebraically as described above, and the resulting concentrations of cupric ion and complex are given in Columns 4 and 5. Of the remaining four solutions, in one pair the pH was the same, but the glycine concentrations were different, while in the other the concentrations of glycine were the same, and those of hydrogen ion different. Since it is not possible to decide, *a priori*, the value of  $r$ , values of  $m$  were calculated for different values of  $r = 0, 1$ , and 2, respectively. From Column 10 it is clear that only when  $r$  is taken equal to 1 is a concordant value, 2, obtained for  $m$ .

The values for the instability constant  $K$  are different for each pair of experiments. This variation is due in the first pair to the uncertainty of the pH values, which were obtained colorimetrically, and in the other solutions is probably due to hydrogen ion

TABLE I  

$$\text{Values of } m, r, \text{ and } K \text{ for } \frac{(\text{Cu}^{++}) (\text{Glycine}^{\pm})^m}{(\text{CuGlycine}_m) (H^+)^r} = K \text{ for First Acid Glycine}$$
  
 Concentration of  $\text{CuSO}_4$  in all solutions = 0.002 M.

Solution No.	pH (2)	Cu electrode E.M.F. (3)	Cu <sup>++</sup> (4)		Cu in complex (5)		Total glycine (6)	Ionization of glycine (7)	Free glycine <sup>±</sup> (8)		Values of <i>m</i> (9)			Log $\frac{K}{r=1}$ (10)	<i>K</i> (11)
			mol	Log	mol	Log			mol	Log	<i>r</i> = 0	<i>r</i> = 1	<i>r</i> = 2		
G 7	1.8*	volt	0.0014 S	-2.85	0.00058 S	-3.24	mol	per cent	mol					+0.15	1.4
G 8	2.0*		0.00084 S	-3.08	0.00116 S	-2.94	0.50	30	0.149	-0.83	3.0	1.8	0.7	+0.20	1.6
G 22	2.24†	+0.0206	0.00020	-3.70	0.00180	-2.74	0.20	44.8	0.088	-1.06	1.9	2.0	2.0	-0.83	0.15
G 23	2.25†	+0.0067	0.00059	-3.23	0.00141	-2.85	0.10	45.4	0.044	-1.86				-0.85	0.14
G 41	1.97†	-0.0048	0.00145	-2.84	0.00055	-3.26	0.40	29.2	0.117	-0.93	4.0	2.5	1.0	+0.51	3.2
G 42	2.27†	-0.0070	0.00058	-3.24	0.00142	-2.85	0.40	46.3	0.185	-0.73	Mean value of <i>m</i> = 2.1			+0.40	2.5

\* = pH determined colorimetrically; error  $\pm 0.2$ .

† = pH determined by glass electrode; error  $\pm 0.02$ .

S = from analysis of absorption spectra.

liquid junction potentials. Since the value of  $m$  is based only on the difference in potential between two members of a pair, it is probably unaffected by this junction potential, which will be nearly the same in each member of the pair. The possibility discussed above, of the existence of still another complex in this pH region, may also be responsible for variations in the value of  $K$ . The agreement previously found between absorption curves indicates that the range of stability within which this other complex exists, if it exists at all, is very narrow.

The present data, therefore, point to the conclusion that the compound whose absorption spectrum is Curve 3 in Fig. 1, and to which the values of  $m = 2$ , and  $r = 1$  apply, is the principal one found in high acidity.

*Second Acid Complex*—The conditions under which the second acid copper-glycine exists are too restricted to allow of any reliable determination of the values of  $m$  and  $r$  by these methods. It appears within only a narrow range of hydrogen ion and glycine concentrations. The minimum variation in glycine concentration necessary for a reliable determination of the value of  $m$  causes this compound to disappear in one or other of the solutions. At pH 6, and with glycine concentrations varying from 0.002 to 0.005  $M$ , the absorption spectra indicated that the principal compound was this second acid complex. The value deduced for  $m$  from a few measurements made on these solutions was between 2 and 3; and the power of  $(H^+)$  was apparently fractional; *i.e.*, 1 H ion was released by the formation of 2 or more complex cupric ions.

The absorption curve of the second acid copper-glycine is nearly identical with that of the second acid copper-alanine. The constitution of the latter compound seems to be firmly established as  $Cu_2alanine_3$ . From this identity the constitution of the second acid copper-glycine may be taken to be similar; *i.e.*,  $Cu_2glycine_3$ . Such a constitution would account for the effect of dilution favoring the second acid compound instead of the neutral complex whose formula is  $Cu\ glycine_2$ , since the compound containing fewer amino acid molecules to 1 of copper would be more stable at lower total amino acid concentrations. This is discussed below in connection with neutral and basic alanine.

*Neutral Complex*—The data and the calculations of  $m$  and  $r$  for the neutral copper-glycine are set out in Table II. The data fall



TABLE  
Calculation of  $m$  for  $\frac{(Cu^{++})(Glycine)_m}{(CuGlycine_m)(H^+)}$   
or Neutral Copper-Glycine

Concentration of  $CuSO_4$  in all solutions = 0.002 M.

Solution No.	$CuSO_4$ in stand. sol.	Cu electrode E.M.F.	Free $Cu^{++}$		Bound Cu		pH	Total ionized glycine	Bound glycine for $m = \frac{1}{2}$	Free ionized glycine		From solution No.	$m$	From solution No.	$m$
			$mols$	Log	$mols$	Log				$mols$	Log				
G 43	0.01	0.056	$1.29 \times 10^{-4}$	4.11	$1.87 \times 10^{-3}$	3.272	5.5* ca.	0.025	$3.7 \times 10^{-3}$	0.021	2.322	G 43 + G 44	2.9	G 45 + G 46	1.9
G 44	0.01	0.0845	$1.38 \times 10^{-4}$	5.14	$1.99 \times 10^{-3}$	3.299	5.5* "	0.05	$3.98 \times 10^{-3}$	0.046	2.663	G 43 + G 45	2.3	G 43 + G 46	2.3
G 45	0.01	0.100	$4.17 \times 10^{-4}$	6.62	$2 \times 10^{-3}$	3.301	5.5* "	0.10	$4 \times 10^{-3}$	0.096	2.982	G 44 + G 45	1.6		
G 46	0.01	0.118	$1.05 \times 10^{-3}$	6.02	$2 \times 10^{-3}$	3.301	5.5* "	0.20	$4 \times 10^{-3}$	0.196	1.292	G 44 + G 46	1.8		
												$r = 1$		$r = 2$	
G 47	0.001	0.130	$4.0 \times 10^{-3}$	3.60	$2 \times 10^{-3}$	3.301	6.13†	0.10	$4 \times 10^{-3}$	0.096	2.982	G 47 + G 48	2.1	G 47 + G 48	1.5
G 48	0.001	0.148	$1.0 \times 10^{-3}$	3.00	$2 \times 10^{-3}$	3.301	6.28†	0.16	$4 \times 10^{-3}$	0.156	1.193				

Mean value of  $m$  from Solutions G 43, G 44, G 45, and G 46 (pH<sub>2</sub> - pH<sub>1</sub> in Solution G 47,  $K = 0.25$ ; in Solution G 48,  $K = 0.23$ ).

by electrometric and colorimetric methods described in the text.  
by glass electrode in solution containing copper.

into two groups. In Solutions G 43 to G 46 the pH was obtained colorimetrically and the cupric ion concentrations given cannot be considered, therefore, as absolute values. However, the differences in potential correspond to actual differences in cupric ion concentration, and the value obtained for  $m$ , which is derived only from these differences, we therefore consider reliable. In Solutions G 47 and G 48 the hydrogen ion concentrations were measured by means of the glass electrode in the final solutions containing copper, and the pH of the standard  $\text{CuSO}_4$  solution was adjusted to 6.1. The data obtained from these two solutions are therefore the most trustworthy. Both groups of data indicate clearly that the value of  $m$  is 2, agreeing with the constitution found for the crystals of neutral copper-glycine in the literature.

On the other hand, these data do not permit of a decision regarding the value of  $r$ , since the hydrogen ion concentrations were maintained constant, or varied only slightly, in order to calculate  $m$ . When  $m = 2$  is substituted in the mass law equation and the data of Solutions G 47 and G 48 are used, a value of 1.1 is obtained for  $r$ , but the pH difference is too small for this value to be relied upon. Larger differences in pH are not experimentally feasible here, since it would then be impossible to estimate the true copper electrode potential difference on account of the varying and unknown hydrogen ion liquid junction potentials. This difficulty was circumvented by measuring the change in hydrogen ion concentration after the addition of  $\text{CuSO}_4$  to an isoelectric solution of the amino acid containing 0.1 M  $\text{K}_2\text{SO}_4$ . The increase in hydrogen ion concentration plus the hydrogen ions absorbed by the glycine corresponds to the number of hydrogen ions released in the formation of the copper complex. The ratio of the number of hydrogen ions released to the number of molecules of copper known to be bound gives the value of  $r$ . The changes in hydrogen ion concentration were measured by means of the glass electrode.

The procedure was as follows: 0.1 M  $\text{CuSO}_4$  was added from a micro burette to 25 cc. of 0.5 M glycine in 0.1 M  $\text{K}_2\text{SO}_4$ , initially present in the glass electrode vessel. After each addition, with continuous, thorough stirring by means of a current of nitrogen, 3 to 5 minutes were allowed for the attainment of a steady potential which did not drift more than 0.3 millivolt during this interval. These potentials were converted to pH values by means of a

graph constructed from the glass electrode potentials of a series of phthalate buffers extending between pH 3.6 and 6.1. The graph obtained was a straight line so that no uncertainty was incurred in interpolation. Its slope at 23° was found to be 57.6 millivolts per unit difference in pH. At the end of the titration, which as a rule occupied about an hour, the constancy of the glass electrode during the titration was checked by measuring again the potential of one or more of the buffer solutions. The change was never more than 0.3 millivolt.

TABLE III

*Number of Equivalents of Hydrogen Ions Set Free in Formation of Neutral Copper-Glycine*

25 cc. of 0.5 M glycine containing 0.1 M  $K_2SO_4$  were initially present.

0.1 M $CuSO_4$ added	Total Cu in solution = total bound Cu	pH	( $H^+$ )	Glycine in undisso- ciated form = bound ( $H^+$ ) $1 - \alpha$	Total ( $H^+$ ) liberated (4) + (5)	Ratio ( $H^+$ ) set free Complex formed $\frac{(6)}{(2)}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
cc.	mol per l.		mol per l.	mol per l.	mol per l.	
0	0	6.04				
0.3	0.00119	4.90	0.0000126	0.00131	0.00132	1.1
0.4	0.00158	4.77	0.0000170	0.00177	0.00179	1.1
0.5	0.00196	4.65	0.0000224	0.00231	0.00233	1.2
0.6	0.00234	4.58	0.0000263	0.00271	0.00274	1.2
0.7	0.00272	4.52	0.0000302	0.00308	0.00311	1.1
0.8	0.00310	4.47	0.0000339	0.00345	0.00348	1.1
1.0	0.00385	4.38	0.0000417	0.00418	0.00422	1.1

The total concentration of bound copper in the neutral complex form (Column 2, Table III) was taken as equal to the amount added. The error so incurred is negligible at this pH, when the glycine, as it is here, is greatly in excess of the copper. The undissociated glycine ( $1 - \alpha$ ) in Column 5 was calculated on the basis of a *Zwitter Ion*  $pK_a$  of 2.33, and a value of  $m$  for the neutral complex of 2. Column 7 shows that 1 hydrogen ion is liberated when 1 cupric ion is bound as neutral copper-glycine. When 1 is used for the value of  $r$ , the instability constant  $K$ , from the data of Solutions G 47 and G 48 in Table II, is 0.24.

*Basic Complex*—We were unable to obtain cupric ion measurements of sufficient reliability at alkalinities at about pH 11 to

attempt determinations of  $m$ ,  $r$ , and  $K$  for the basic complex. The following facts can be deduced about it, however.

First, the peak of absorption is still in the orange, though shifted some 400 Å. towards the red (*cf.* Curves 5 and 6, Fig. 1). This may be taken to indicate that whatever inner complex structure is assigned to the neutral complex, that assigned to the basic complex cannot be very different in type, the difference being probably quantitative rather than qualitative. Secondly, the ratio of concentrations of neutral and basic complexes present in a mixture, at any one pH, is not largely influenced by changing the

TABLE IV

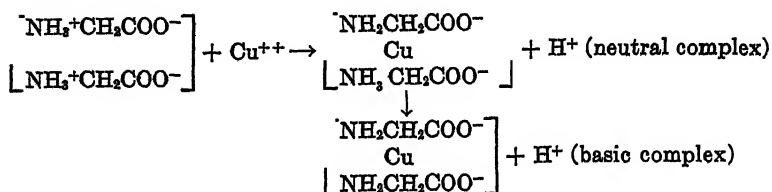
*Dependence of Relative Amounts of Neutral and of Basic Copper-Glycine on Hydrogen Ion Concentration*

Solution No.	pH	Total glycine	Per cent total glycine in		Per cent total Cu as	
			$\begin{array}{c} \text{NH}_3^+ \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{COO}^- \\ \text{form} \end{array}$	$\begin{array}{c} \text{NH}_2 \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{COO}^- \\ \text{form} \end{array}$	Basic copper-glycine	Neutral copper-glycine
		<i>mol per l.</i>				
1	8.05	0.5	98	2	94	6
2	8.1	0.05	98	2	99	1
3	8.1	0.002	98	2	100	0
4	8.7	0.5	92	8	85	15
5	8.7	0.05	92	8	90	10
6	9.2	0.1	78	22	79	21
7	9.8	0.5	47	53	41	59
8	10.2	0.5	27	73	29	71
9	11.1	0.5	4	96	12	88

concentration of amino acid (see Solutions 2 to 5, Table IV). This fact indicates that the number of molecules of glycine per molecule of copper is the same in both these complexes. The third point is that, within the limits of accuracy of the pH determinations and absorption measurements involved, the ratio of concentrations of basic and neutral complexes present in a mixture is the same as the ratio of concentrations of basic and neutral forms of glycine.

In Table IV the amounts of the two complexes were obtained by algebraic treatment of the absorption curves, and the proportion of glycine in the basic form is calculated from the pK of 9.75.

It is clear that these proportions agree with the proportions of the basic complex present. In the absence of confirmatory data, therefore, the simplest interpretation would be as follows: the neutral complex is formed from 2 molecules of glycine and 1 cupric ion, with elimination of 1 H ion; the basic complex is formed from this by elimination of a 2nd H ion. That is



This formulation agrees with the finding that the equilibrium between these two complexes is controlled only by the pH and is uninfluenced by the amino acid concentration. The absorption spectrum to be expected would still be of the same general type, though with some change corresponding to the change in charge.

#### IV. Cupric Salts of Alanine

##### (a) Absorption Spectra

As with glycine, the absorption curves indicated the existence of four copper-alanine complexes, two in the acid, one in the neutral, and one in the alkaline range of pH. In Fig. 3 the absorptions in the visible spectrum of the four compounds are set out and compared with those of copper sulfate (*i.e.* cupric ion), undissociated copper acetate, and cupriammonium sulfate.

In acid solutions the absorption due to the complex was obtained, as with glycine, by determining the free cupric ion with the copper electrode and subtracting the absorption due to this. The extinction coefficients were then computed, the concentration of complex being that of the total copper minus the free cupric ion. The absorption for the first acid complex was derived from the last four sets of measurements in Table V, the absorption spectra of these solutions being taken at the same time. The curve for the second acid complex was obtained from solutions in which the pH varied from 2.0 to 4.0 and the alanine concentration from 0.005 to 0.5 M.

The absorption curve for the neutral complex is the mean from a large number of solutions in which it was clear that only this complex was present. Twelve solutions, in which the pH varied from 6.5 to 11.1 and the alanine concentration from 0.5 to 0.07 M, gave practically identical absorptions. Fig. 4, which is the alanine analogue of Fig. 2, shows the change in absorption with pH, at constant concentrations of copper and alanine, and also shows the relative constancy of the absorption in the neutral range.

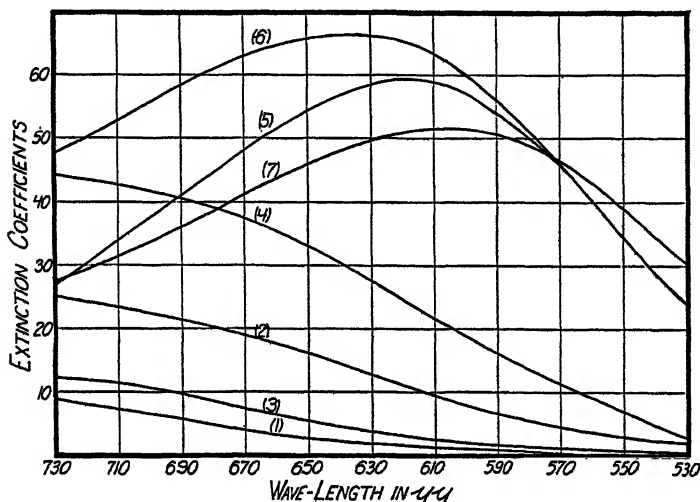


FIG. 3. Absorption curves of cuprialanine compounds. Extinction coefficients,  $\log \frac{I_0}{I} \cdot \frac{1}{c} \cdot \frac{1}{L \text{ (cm.)}}$  at various wave-lengths in  $\mu\mu$ . Curve 1, copper sulfate; Curve 2, copper acetate in alcohol; Curve 3, first acid cuprialanine; Curve 4, second acid cuprialanine; Curve 5, neutral cuprialanine; Curve 6, basic cuprialanine; Curve 7, cupriammonium sulfate.

Curve 6 in Fig. 3 is the mean of the twelve curves thus obtained. With this curve and those of the first and second acid complexes, together with the curve for  $\text{CuSO}_4$ , it was possible to account for the absorptions of all the other solutions in the acid range up to pH 6.8, and with alanine concentration from 0.002 to 0.5 M, the copper being always constant at 0.002 M.

In alkaline solutions the change in absorption with pH was different from that found with glycine. As Fig. 4, B shows, with

alanine 0.1 M very little increase in absorption occurs even up to pH 11; i.e., the basic compound either has an absorption curve similar to that of the neutral compound or else is not being formed at this dilution. The latter alternative is the true explanation, for at pH 11.1 the absorption in the red steadily increased as the

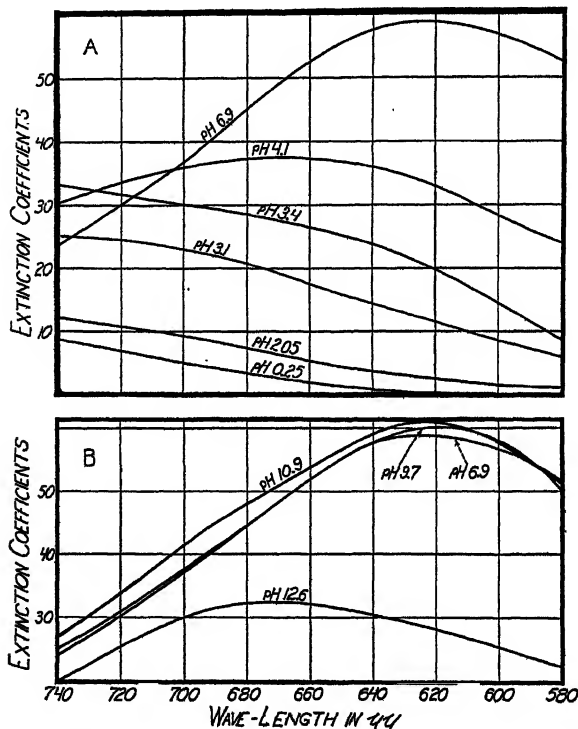


FIG. 4, A. Effect of changing H ion concentration on the absorption of cuprialanine solutions containing the same amounts of copper and of alanine; Cu = 0.002 M, alanine = 0.10 M; acid range.

FIG. 4, B. Same as Fig. 4, A; alkaline range.

alanine concentration was increased. Fig. 5 shows this change from a 7 times excess of alanine over copper concentration—which gives a curve almost identical with that of the neutral complex—to a 500 times excess. The absorption of the solution containing 500 times excess alanine over copper was the same as that of a solution containing 0.001 M copper and 0.9 M alanine,

*i.e.* a 900 times excess, at the same pH. This curve was therefore considered to be that of the pure basic complex. Its accuracy was corroborated by the concordant results obtained when it was employed, with the absorption curve of the neutral copper-alanine, in the analysis of the absorption of all other solutions in the alkaline range. In extreme alkalinity, as with glycine, precipitation occurred, and the absorption was correspondingly lowered.

Comparison of Figs. 4, *A* and 4, *B* with Figs. 2, *A* and 2, *B* shows that the change of absorption with pH in the acid ranges is very similar with alanine and glycine, while in the alkaline range

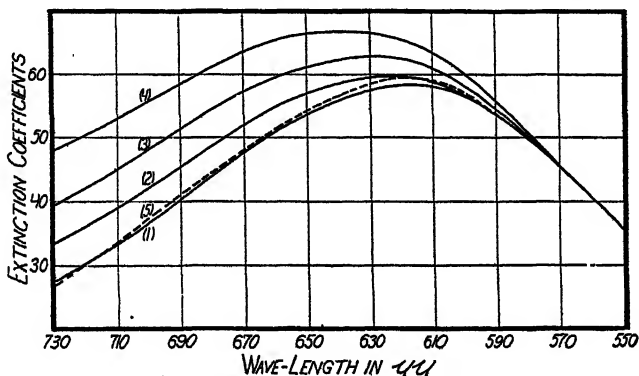


FIG. 5. Effect of increasing alanine concentration at constant pH = 11.1, Cu = 0.002 M. Curve 1, alanine 0.016 M; Curve 2, alanine 0.20 M; Curve 3, alanine 0.50 M; Curve 4, alanine 1.0 M; Curve 5, neutral cupri-alanine (*cf.* Fig. 3).

a considerable difference is noticeable. The significance of this is further discussed below.

#### (b) Potentiometric Data and Constitution

*First Acid Copper-Alanine*—The values of  $m$  and  $r$  for this complex were obtained from the data in Table V. A concordant and reasonable value for  $m$  was obtained only when  $r$  was taken equal to 0;  $m$  is then clearly 2. This complex therefore differs from the first acid copper-glycine, in which 1 hydrogen ion is released. A corresponding difference appears in the absorption curves of these two complexes. The curve of cupric alanine, for



TABLE V

Calculation of  $m$ ,  $r$ , and  $K$  for  $(Cu^{++}) \frac{(Alanine)^m}{(CuAlamine_m)(H^+)^r} = K$  for First Acid Copper-Alanine

Solution No.	Total CuSO <sub>4</sub>	Cu <sup>++</sup> in standard solution	Cu electrode E.M.F.	Free Cu <sup>++</sup>		Bound Cu		pH	Total alanine ionized	Bound alanine for $m = 2$	Free ionized alanine		From solution No.	$m$ $r = 0$	$K \times 10^4$
				Log	mol	Log	mol				mol	Log			
A 20	0.002	0.001	soli	4.82	0.00134	3.127	2.05*	0.1	0.034	0.003	0.031	2.491	A 20 + A 21	2.1	4.7
A 21	0.002	0.0005 S		4.699	0.0015	3.176	2.5*	0.1	0.040	0.003	0.037	2.568			4.6
A 34	0.002	0.00126	-0.0031	3.10	0.00074	4.869	1.40†	0.25	0.028	0.001	0.027	2.431	A 34 + A 33	1.8	12
A 33	0.002	0.001	+0.010	4.66	0.00154	3.188	1.97†	0.25	0.075	0.003	0.072	2.857	A 33 + A 31	2.2	15
A 31	0.002	0.001	+0.030	5.98	0.00191	3.281	1.99†	0.5	0.16	0.004	0.16	1.204	A 34 + A 31	2.0	14
A 32	0.002	0.001	+0.014	4.52	0.00167	3.22	1.51†	0.5	0.065	0.003	0.062	2.792	A 34 + A 32	2.2	8

S = from analysis of spectrophotometric data only.

\* = determined by means of hydrogen electrode and colorimetrically as described in text.

† = determined by means of glass electrode.

TABLE V.

Calculation of  $a$ , and  $K$   $\frac{Cu^{++}}{CuAlanin} - \frac{m}{H^+} = K$  for  $S_4$  Acid Copper-Alan.

Solution No.	Total CuSO <sub>4</sub>	Cu <sup>++</sup> in standard solution	Cu electrode E.M.F.	Free Cu <sup>++</sup>		Bound Cu		pH	Total alanine ionized	Bound alanine $a = 2$	Free ionized alanine		From solution No.	$m$ $r = 1$ $a = 2$	$K \times 10^7$
				Log	mol	Log	mol				mol	Log			
A 103	0.002	0.001	-0.0070	3.235	0.00172	4.447	4.00†	0.0025	0.00245	0.00042	0.00203	3.308	A 103 + A 104	2.7	9
A 104	0.002	0.001	-0.0051	3.170	0.00143	4.716	3.88†	0.004	0.00388	0.00078	0.00310	3.491	A 104 + A 106	3.3	9.5
A 106	0.002	0.001	-0.0036	4.88	0.00124	3.093	3.92†	0.008	0.00776	0.00186	0.00590	3.771	A 103 + A 106	3.1	9

which  $r = 0$ , is very low in the visible spectrum, resembling the curve of  $\text{CuSO}_4$ , while that for the copper-glycine, for which  $r = 1$ , is much higher. The former approximates the curve of a simple salt; the latter that of a "complex."

The values of  $K$  derived from spectrophotometric data (Solutions A 20 and A 21, Table V) are distinctly lower than those derived from potentiometric measurements. Probably, as in the case of glycine, the discrepancy is due to the hydrogen liquid junction potentials. However, the variation in the value of  $K$ , in view of the experimental difficulties in these solutions, is not great, and its mean value may be taken to be close to  $1 \times 10^{-3}$ .

TABLE VII

*Calculation of Number of Hydrogen Ions Released in Formation of Second Acid Copper-Alanine*

Ala- nine  (1)	pH after addition of 0.002 M $\text{CuSO}_4$ (2)	( $\text{H}^+$ )  (3)	Log $\frac{1-\alpha}{\alpha} =$ $\text{p}K - \text{pH}$ (4)	Fraction of alanine undis- sociated $1-\alpha$ (5)	( $\text{H}^+$ ) bound by alanine (6)	Total ( $\text{H}^+$ ) released (3) + (6) (7)	Total (Cu) bound (8)	Ratio ( $\text{H}^+$ ) set free Cu in complex $\frac{(7)}{(8)}$ (9)
<i>mol</i> <i>per l.</i>		<i>mol per l.</i>			<i>mol per l.</i>	<i>mol per l.</i>	<i>mol per l.</i>	
0.0020	3.98	0.000105	2.35	0.022	0.000044	0.000149	0.00038	0.4
0.0025	3.99	0.000099	2.34	0.022	0.000055	0.000154	0.00028	0.6
0.0040	3.99	0.000102	2.34	0.022	0.000088	0.000190	0.00049	0.4
0.0040	3.90	0.00013	2.43	0.026	0.000104	0.000234	0.00052	0.5
0.0050	3.94	0.00012	2.39	0.024	0.00012	0.00024	0.00054	0.5
0.0080	3.80	0.00016	2.53	0.033	0.000261	0.000421	0.00124	0.4
0.0100	3.84	0.00014	2.49	0.030	0.00030	0.00044	0.00085	0.5

*Second Acid Complex*—The second acid alanine complex presented the same difficulty as the corresponding glycine compound; *i.e.* the restricted range of concentrations of amino acid and of hydrogen ion in which this form exists free of other complexes. After a number of trials a few solutions containing only this compound in equilibrium with cupric ions were obtained. The resulting data are given in Table VI. Concordant values for  $m$ ,  $r$ , and  $K$  were obtained only when the reaction was taken to be



(*i.e.*,  $m$  has the value 1.5, or is 3 if  $a = 2$ , where  $a$  is the number of Cu atoms in the complex. Also, if  $a = 2$ ,  $r = 1$ ).

Further corroboration of this formula is found in the results of direct estimation of the hydrogen ions set free when this complex is formed (Table VII). The hydrogen ion activities in these solutions were measured by means of the glass electrode, free cupric ion concentrations by means of copper electrode potentials. Spectrophotometric measurements showed that the major part of the bound copper was in the form of the second acid complex. The ratio of the numbers of hydrogen ions set free to cupric ions bound (Column 9) is clearly 0.5; *i.e.*, if  $a = 2$ ,  $r = 1$ .

The absorption curve of the second acid copper-alanine is nearly identical with that found by trial and error for the corresponding copper-glycine. This coincidence suggests, as pointed out above, that the constitution of the latter compound is similarly  $\text{Cu}_2\text{glycine}_3$ . The findings, mentioned above, that for the second acid glycine complex,  $r$ , the power of  $(\text{H}^+)$ , appeared to be fractional and that  $m$  appeared to be of the order of 2 or 3, also support this formula. In the absence of other evidence, we have therefore assumed it to be correct.

*Neutral Copper-Alanine*—The electrometric data for the neutral complex (Table VIII) fall into two groups: those derived from solutions at pH about 6.8, and those at pH 7.9. The hydrogen ion liquid junction potentials are again probably responsible for the discrepancy in the values of  $K$ . The value of  $m$  is either 2.5 or 3; *i.e.*, the complex has the constitution  $\text{Cu alanine}_3$  or  $\text{Cu}_2\text{alanine}_5$ . In either case, the direct estimation of the hydrogen ions set free in the formation of this compound, given in Table IX, shows unequivocally that 1 hydrogen ion is set free for each cupric ion bound. In this respect the complex resembles the corresponding copper-glycine. The power of  $(\text{H}^+)$  was accordingly taken as 1 in the calculation of  $m$  in Table VIII. The absorption curve of this complex is somewhat higher than that of the neutral copper-glycine, though the maximum is in nearly the same part of the spectrum. A possible explanation for these facts is that the higher absorption of neutral copper-alanine is due to the larger number of molecules of amino-acid relative to copper in its composition, but that the number of nitrogen atoms attached to copper, which according to Kober and Haw governs the position of the peak of absorption, is the same in the two compounds.

*Basic Copper-Alanine*—The basic alanine complex is much

TABLE VIII

Calculation of  $\alpha$ ,  $m$ , and  $r$  for  $\frac{(Cu^{++})^{\alpha} (Alanine)^m}{(Cu_0 Alanine_m) (H^+)^r} = K$  for Neutral Copper-Alanine

Solution No.	Cu <sup>++</sup> in stand-ard solution	Cu electrode E.M.F.	Free Cu <sup>++</sup>		Bound Cu		pH	Total alanine (all ion-ized)	Bound alanine m = 3	Free alanine (all ionized)		From solution No.	m r = 1	Log K		
			mols	Log	mols	Log				Log	Log			m = 3 m = 2.5		
A 114	10 <sup>-4</sup>	+0.100	4.17 × 10 <sup>-8</sup>	8.62	0.002	3.301	6.71†0.4	mol	mol	0.006	0.394	1.596	A 114 + A 115	3.3	0.8	1.0
A 115	$\left\{ \begin{matrix} 10^{-3} \\ 10^{-4} \end{matrix} \right.$	$\left\{ \begin{matrix} +0.0835 \\ +0.0728 \end{matrix} \right.$	3.47 × 10 <sup>-7</sup>	7.54	0.002	3.301	6.81†0.2			0.006	0.194	1.288	A 114 + A 116	3.0	0.9	1.3
A 116	10 <sup>-4</sup>	+0.0427	2.0 × 10 <sup>-8</sup>	8.30	0.002	3.301	6.89†0.1			0.006	0.094	2.973	A 115 + A 116	2.6	0.8	1.3
A 117	10 <sup>-5</sup>	+0.156	5.37 × 10 <sup>-11</sup>	11.73	0.002	3.301	7.91†0.25			0.006	0.244	1.387	A 117 + A 118	2.5	-1.5	-1.2
A 118	10 <sup>-5</sup>	+0.110	4.47 × 10 <sup>-9</sup>	9.28	0.002	3.301	7.93†0.0625			0.006	0.0565	2.752	A 117 + A 119	2.5	-1.8	-1.2
A 119	10 <sup>-5</sup>	+0.050	2.04 × 10 <sup>-7</sup>	7.31	0.002	3.301	7.89†0.0156			0.006	0.0096	3.982	A 118 + A 119	2.6	-2.1	-1.1

† = pH measurements made with glass electrode in final mixtures containing copper.

more sensitive to changes in the concentration of amino acid than is the basic glycine compound. At pH 11.1, increase in the concentration of alanine from 0.2 to 1.0 M changed the amount of the basic complex (by analysis of the curves in Fig. 5) from 25 to 100 per cent of the total copper. This indicates that the number of

TABLE IX

*Number of Equivalents of Hydrogen Ions Set Free on Formation of Neutral Copper-Alanine*

Experiment I. 25 cc. of 0.4 M alanine containing 0.1 M  $K_2SO_4$ .

" II. 25 " " 0.5 " " " 0.1 " "

Experiment No.	CuSO <sub>4</sub> added	Cu in complex formed	pH	(H <sup>+</sup> )	Fraction of alanine undissociated $1 - \alpha$	Alanine undissociated	Total (H <sup>+</sup> ) set free (5) + (7)	Ratio (H <sup>+</sup> ) set free Complex formed
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	cc. 0.1 M	mol per l.		mol per l.		mol per l.	mol per l.	
I	0	0	6.08					
	0.20	0.00079	5.02	0.0000096	0.00204	0.000813	0.000823	1.04
	0.30	0.00118	4.85	0.0000141	0.00307	0.00120	0.00121	1.03
	0.40	0.00157	4.72	0.0000191	0.00407	0.00162	0.00164	1.04
	0.50	0.00196	4.62	0.0000240	0.00510	0.00203	0.00205	1.04
	0.60	0.00234	4.54	0.0000289	0.00613	0.00242	0.00245	1.05
	0.70	0.00272	4.46	0.0000347	0.00735	0.00290	0.00293	1.07
	0.80	0.00310	4.41	0.0000389	0.00826	0.00324	0.00328	1.06
	1.00	0.00384	4.33	0.0000468	0.01000	0.00385	0.00390	1.02
II	0.30	0.00118	5.07	0.0000085	0.00182	0.00091	0.00092	0.78
	0.40	0.00157	4.88	0.0000130	0.00281	0.00138	0.00139	0.89
	0.50	0.00196	4.76	0.0000174	0.00370	0.00181	0.00183	0.93
	0.60	0.00234	4.67	0.0000214	0.00455	0.00221	0.00223	0.95
	0.70	0.00272	4.60	0.0000251	0.00534	0.00260	0.00263	0.97
	0.80	0.00310	4.55	0.0000282	0.00599	0.00292	0.00295	0.95
								Mean 0.99

molecules of alanine bound to 1 of copper is not the same in the neutral and basic compounds. From the expressions

$$\frac{(\text{Cu})(\text{alanine})^s}{(\text{Cu alanine}_s)(\text{H}^+)^r} = K_s \text{ for the neutral complex}$$

and

$$\frac{(\text{Cu})(\text{alanine})^v}{(\text{Cu alanine}_v)(\text{H}^+)^{r''}} = K_b \text{ for the basic complex}$$

it follows that at any one pH, where both complexes are present, the term for Cu ion vanishes, and the expression

$$\frac{(\text{Cu alanine}_y)}{(\text{Cu alanine}_x)} = k \cdot (\text{alanine})^{y-x} \cdot (\text{H}^+)^{r'-r''}$$

is obtained, where  $k = \frac{K_n}{K_b}$ . Hence at constant pH, if  $y$  is greater

than  $x$ , the proportion of basic to neutral complex will become greater with increasing alanine concentration. This is, in fact, what occurs, so that the value of  $m$  for the basic copper-alanine is therefore greater than 2.5 or 3, according to which figure is taken for the neutral complex. In the case of glycine the distribution of copper between neutral and basic complexes was shown above to be almost independent of amino acid concentration, and hence it was concluded, by similar reasoning, that the number of molecules of glycine bound by 1 of copper is the same in the neutral as in the basic complex.

## V. DISCUSSION

### (a) *Constitution of Complexes*

The properties of the eight complexes described here are summarized in Table X, with their ranges of stability, empirical formulæ, and references to the data. We have not set down definite structural formulæ because we feel that the data so far obtained are insufficient. Nevertheless, certain deductions may be made regarding their structure from the characteristic absorption spectra of the four types of compounds formed, on the basis (a) of the rule proposed by Kober and Haw, and (b) of a second rule suggested by our observations, that the height of an absorption curve, without reference to its position in the spectrum, is greater the larger the number of amino acid molecules in the complex.

The spectra of the first acid complexes show a low absorption in the visible portion limited to the red and orange (Figs. 1 and 3). By application of the above rules, the copper in these compounds therefore is linked only to the oxygen atoms of the carboxyl groups; *i.e.*, they approximate undissociated salts. The peaks of their curves are presumably far in the infra-red. The maximum absorption of the second acid complexes, on the assumption that

TABLE X  
Summary of Copper Complexes of Glycine and Alanine

Complex (1)	Absorption (2)	Formula (3)	Range of stability (4)	Probable formula of complex (5)	H ions set free per atom Cu bound (6)	Absorption characteristics (7)
1st acid glycine.....	Fig. 1	Table I	Cu. pH 0.5-2.5	Cu glycine <sub>2</sub>	1	Curve similar to Cu- (OAc) <sub>2</sub> in alcohol
2nd " .....	" 1		pH 2-5; in high dilu- tion, up to pH 7	Cu glycine <sub>3</sub>	0.5	Peak just in infra-red
Neutral glycine.....	" 1	Tables II, III	pH 5-8; overlapping basic complex to pH 10.5	Cu glycine <sub>2</sub>	1	" at 6250 Å.
Basic " .....	" 1	Table IV	pH 8-12; not affected by dilution	Cu glycine <sub>2</sub>	2	" " 6700 "
1st acid alanine .....	" 3	" V	Cu. pH 0.5-2.5	Cu alanine <sub>2</sub>	0	Very low absorption; about half that of 1st acid glycine and close to that of cu- pric ion
2nd " .....	" 3	Tables VI, VII	pH 2.5-6; favored by dilution	Cu alanine <sub>3</sub>	0.5	Exactly similar to 2nd acid glycine
Neutral " .....	" 3	Tables VIII, IX	pH 5-9; in dilute solu- tions to pH 11	Cu alanine <sub>2</sub> or Cu <sub>2</sub> alanine <sub>3</sub>	1	Peak at 6200 Å.; higher than neutral glycine
Basic " .....	Figs. 3, 5		pH 8-11; only in con- centrated solutions	Cu alanine <sub>2</sub>	2	Peak at 6460 Å.; higher than basic glycine

the form of all the curves is the same, may be taken to be in the very near infra-red. This suggests the first appearance of a Cu—N bond, which the liberation of 1 hydrogen for every 2 cupric ions bound also indicates. In the case of both neutral complexes 1 hydrogen ion is released for every cupric ion bound. The number of Cu—N bonds of this type is therefore twice as great as in the second acid compounds, which is in accord with the observed shift of the absorption maximum to approximately 6200 Å.

As indicated in Column 5, Table X, an unexpected difference occurs between glycine and alanine in the number of amino acid molecules bound per atom of copper in the neutral and probably also in the basic complexes. Tentatively we have interpreted the higher absorption of the alanine compounds to be due to the larger number of amino acid molecules in the complex.

#### *(b) Application to Donnan Equilibria*

It is probable that the variety of complexes formed by protein with bi- and polyvalent cations is at least as great as that found between copper and glycine and alanine. This phenomenon complicates the calculation of Donnan equilibrium relations with proteins. Since the type and extent of complex formation as in the case of amino acids probably varies throughout the whole pH range usually studied, a continually varying correction is necessary for the ions bound in undissociated complexes to the protein. This must be one of the factors responsible for the divergence of the values for the ratios of the calcium concentrations on two sides of a semipermeable membrane from those calculated by means of the unmodified Donnan equation when protein is present on one side (20). Complex formation probably accounts also for the findings of Pincus and Kramer (21) who showed that though the distribution of the monovalent ions between cerebrospinal fluid and serum was in accord with the Donnan equilibrium, the calcium distribution was markedly different.

There are indications that this complication of simple Donnan equilibrium relations by complex formation occurs even with monovalent ions (22). Since complex formation occurs least in highly acid solutions where ionization of the acid groups is suppressed, it would be expected that in this zone simple Donnan equilibrium relations would hold best. This has, in fact, been observed by



Bonino and Garello (23) in their study of Donnan equilibria between ovalbumin and cobaltous salts. At low pH values, the Donnan relation was found to be obeyed; as neutrality was approached, the divergence of the ion concentrations from the calculated values became progressively greater.

#### SUMMARY

1. The equilibrium relations existing in solution at room temperature between cupric ions and glycine and alanine have been studied by measurement of the absorption in the visible spectrum, and by copper electrode potentials, through a range of hydrogen ion concentrations from pH 0 to 13.

2. The variations in absorption indicate the existence of at least four types of complexes with both glycine and alanine. According to the range of hydrogen ion concentration in which each predominates they have been designated as first acid, second acid, neutral, and basic complexes. The approximate ranges of hydrogen ion and relative amino acid concentrations, in which each type of complex predominates, are delineated.

3. The absolute absorption spectra, in the visible region, of each of the eight complexes in a pure state have been determined. In some cases these were obtained directly, in others by deduction from mixtures. From these curves the absorption spectra of all the mixed systems examined could be derived.

4. The constitution of these eight compounds has been deduced from potentiometric and spectrophotometric data. Of these the constitutions of the second acid glycine, neutral alanine, and basic alanine are uncertain.

5. The number of hydrogen ions set free in the formation of all except the two basic compounds was estimated. The orders of magnitude of the instability constants of the first acid and neutral copper-glycines and of the first acid, second acid, and neutral copper-alanines were determined.

6. The significance is discussed of the phenomena described here in Donnan equilibria involving proteins.

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# EFFECT OF HEAT AT VARYING CONCENTRATIONS OF HYDROGEN ION ON VITAMIN B (B<sub>1</sub>) IN PROTEIN-FREE MILK\*

BY NELLIE HALLIDAY

WITH THE ASSISTANCE OF VEDA ELLEN HILLER

*(From the Section of Home Economics, Michigan Agricultural Experiment  
Station, East Lansing)*

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Vitamin B (old nomenclature) has long been known to be relatively sensitive to heat, particularly in an alkaline medium. As the evidence for the existence of at least two vitamins grew, it seemed apparent that, in yeast extracts particularly, the antineuritic vitamin was the less stable of the two when the extract was heated. This has led to the use of the terms "heat-labile antineuritic" and "heat-stable growth-promoting" vitamins for vitamins B (B<sub>1</sub>) and G (B<sub>2</sub>) respectively.

A full review of the literature will not be included in the present paper inasmuch as Sherman and Smith (1) have adequately brought together the material up to the end of 1931. A few references will be mentioned, however, which show that the stability of vitamin B appears to vary in different media, as has been shown to be the case with vitamin G (B<sub>2</sub>).

As early as 1917 Chick and Hume (2) found that wheat embryo could be heated 2 hours at about 100° without significant loss. However, yeast extract lost about 50 per cent of its potency on being heated 1 hour at 100° and about 70 per cent if heated 2 hours at 122°. At 118–124° the vitamin content of wheat embryo was reduced about 75 to 90 per cent on 2 hours of heating. Pigeons were used as the experimental animals and the results, therefore, refer to the antineuritic vitamin.

In 1920 Emmett and Luros (3) showed that the antineuritic

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and growth-promoting vitamins in unmilled rice, as measured by polyneuritic pigeons and young rats respectively, were fairly stable at temperatures around 100–105°, while at higher temperatures the antineuritic vitamin appeared to be less stable to heat and alkali than the growth-promoting vitamin.

In the early work on the differentiation of vitamins B ( $B_1$ ) and G ( $B_2$ ) it was found that autoclaving of yeast would destroy vitamin B, leaving a potent source of the second factor. Bisbey (4) has shown, however, that a brewers' yeast rich in vitamin B, even after 6 hours of autoclaving under 0.1 M KOH, still contained such amounts of the antineuritic vitamin that the material could not be used in a diet where a source of vitamin G was desired, free from vitamin B.

Sherman and Spohn (5) found no measurable destruction of "vitamin B" in milk powder when heated dry with free access to air at 100° for 48 hours. In tomato juice Sherman and Burton (6) found the extent of destruction to be dependent upon the hydrogen ion concentration employed. These two experiments were conducted on the vitamin B complex, but since a lack of the antineuritic vitamin becomes apparent before that of vitamin G, it is still possible to evaluate these results in terms of vitamin B.

Guha and Drummond (7) have shown that at pH 1 a vitamin  $B_1$  (B) concentrate can be boiled for 24 hours without any appreciable destruction, at pH 5 it is half destroyed, while at pH 9 it is half destroyed in 1 hour. Shorter periods of boiling resulted in correspondingly less destruction.

These varying results have made it appear that the medium as well as the hydrogen ion concentration, time, and temperature are all factors in the destruction by heat. It seemed of interest therefore to study quantitatively the comparative effect of heat (for varying lengths of time and at varying concentrations of hydrogen ion) on the two vitamins (vitamins B and G) in a protein-free milk preparation. This material is easily prepared, is well standardized, is readily eaten by the experimental animals, and is closely related to natural foods.

#### EXPERIMENTAL

The plan of the work was to determine simultaneously the vitamin B ( $B_1$ ) and G ( $B_2$ ) values of (a) skim milk powder, (b)

protein-free milk (prepared from this skim milk powder) at its natural acidity (about pH 4.3) and after it had been brought to pH 7 and to pH 10, and (c) these solutions after 1 or 4 hours of heating in a water bath at about  $97^{\circ} \pm 1^{\circ}$ .

The effect of heat on vitamin G ( $B_2$ ) at these varying concentrations of hydrogen ion and for varying lengths of time has been reported (8). Full details of the method of preparation of the materials and of the experimental procedure were given.

In a recent contribution from this laboratory (9) it was shown that in the experiments to determine the vitamin B ( $B_1$ ) value of these materials, the animals almost uniformly ceased to grow at as good a rate after about the 5th week of the experimental period, even though they were receiving an adequate amount of vitamin G (15 per cent of the basal diet as autoclaved yeast) and presumably a fair allowance of vitamin B, as evidenced by the initial growth. It seemed evident that some factor was lacking, and should be supplied, in order to make a quantitative determination possible. It was further shown that whole wheat contained a fair allowance of some factor (or factors) which would induce gain in these animals after the weight had remained stationary for several weeks.

As a result of these findings the first work was repeated with this variation. All animals received, in addition to the skim milk powder or liquid supplement, 0.2 gm. daily of whole wheat.

All animals came from mothers which had received either the Sherman and Campbell Diet B (10), or a modification of the Steenbock stock diet. Hartley (11) has shown that no differences in growth were obtained in young rats of very different nutritional history when they received the Chase and Sherman (12) vitamin B-free basal diet plus graded amounts of skim milk powder or tomato juice. In these present experiments no difference was noted in the response of the two groups used. Further, the litters were so divided, after the depletion period, that no two litter mates received the same supplementary feeding. The sex and weight of the animals were matched as carefully as possible. The animals were placed on the experimental diet at 28 to 29 days of age. With very few exceptions the weight was between 35 and 55 gm. After depletion, animals weighing less than 100 gm. and more than 50 gm. were used for experimental purposes. The average weight

of the groups receiving each supplement was about 75 gm. At least six rats were used in each group, but animals which refused to eat the supplements were discarded. All portions were fed daily, except Sunday, in amounts corresponding to a weight of skim milk powder, the vitamin content of which had been determined.

### *Results*

In Fig. 1 are shown curves representing the average growth of experimental animals receiving the vitamin B- ( $B_1$ ) deficient diet plus graded portions of skim milk powder or the various liquid supplements.

The flattening of the growth curves after about the 5th week is noticeable in almost every group. If comparisons are to be made of the vitamin B value of these supplements, it is evident that only the growth during the first 4 or 5 weeks can be considered. Such data are given in Table I.

The solutions at pH 10 show little or no vitamin potency, except when fed in amounts corresponding to 1 gm. of skim milk powder daily. In the case of the unheated solution fed in this amount half of the animals became polyneuritic and died in about 5 weeks. The remaining animals grew at a slow but fairly constant rate throughout the 8 week period. Since the curve represents the average growth of surviving animals, this growth becomes evident during the last few weeks. It is known that animals receiving only a small allowance of the vitamin show greater variation than those receiving a lower or higher level of supplement. These results are included, therefore, as an indication of the variation which may be found, but no other group showed such a lack of uniformity.

In the experiments to determine the vitamin G ( $B_2$ ) value of these same supplements, it was found that the hydrogen ion concentration of the solution at pH 10 (originally) dropped to about pH 8 after 1 hour of heating, and that there was less destruction of the vitamin in this solution than in the unheated material. The results in Series I are such that conclusions as to the percentage destruction are hardly justifiable. It would seem, however, that the degree of destruction in the two solutions (unheated and heated 1 hour) was of the same order of magnitude.

In Fig. 2 are shown the results of the experiments of Series II.

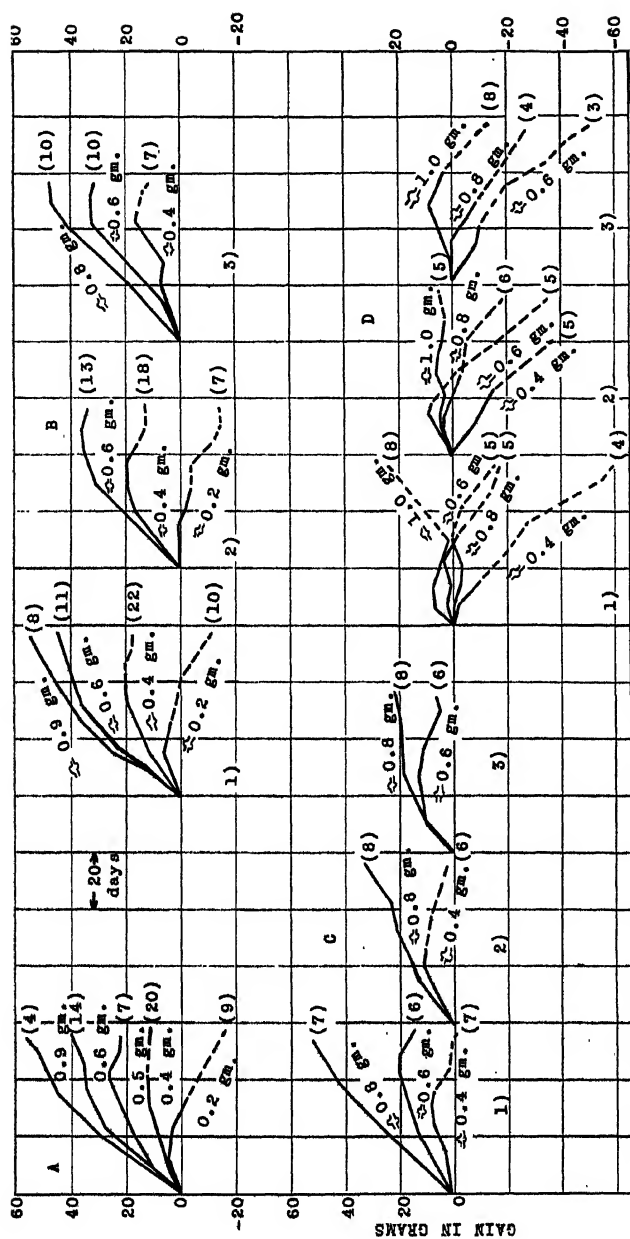


Fig. 1. Average growth curves of rats which received the Chase and Sherman vitamin B- (B<sub>1</sub>) deficient diet plus graded portions of skim milk powder (Group A), graded portions of protein-free milk preparations at pH 4.3, 7, and 10 respectively (Groups B, C, and D). Subgroups 1, 2, and 3 in each case refer to the unheated solutions, solutions heated 1 hour, and those heated 4 hours. The figure at the end of each curve indicates the number of animals of which results are included. The broken line indicates that one or more rats have died, and the curve from this date represents results of the surviving animals.



In this case litter mates received the same basal diet and the same skim milk powder or protein-free milk supplements, and in addition 0.2 gm. of ground whole wheat daily. One animal in each litter received a liquid supplement only, as a check on the earlier work, and these results are included in Fig. 1.

TABLE I

*Showing Relative Vitamin B ( $B_1$ ) Potency and Percentage Destruction, in Terms of Skim Milk Powder Taken As Standard, of Various Protein-Free Milk Preparations*

For comparison are included the vitamin G ( $B_2$ ) values of these same materials, as reported in an earlier contribution.

Supplement	Vitamin B ( $B_1$ )				Vitamin G ( $B_2$ )	
	Series I*		Series II		Potency	Destruction
	Potency	Destruction	Potency	Destruction		
	per cent	per cent	per cent	per cent	per cent	per cent
Skim milk powder.....	100		100		100	
Protein-free milk						
pH 4.3, unheated.....	100	None	90	10	Equal	None
" 4.3, 1 hr.....	90-100	0-10	70-80	20-30	90	10
" 4.3, 4 hrs.....	85	15	60	40	70	30
" 7, unheated.....	85	15	90-100	10	85	15
" 7, 1 hr.....	70	30	70	30	67	30
" 7, 4 hrs.....	50-60	40-50	50-70	40	50	50
" 10, unheated.....	10-25	75-90	20-30	70-80	25-30	70-75
" 10, 1 hr.....	10-25	75-90	20-30	70-80	60	40
" 10, 4 hrs.....	10-25	75-90	20-30	70-80	30-35	65-70

\* Series I refers to the original experiments in which skim milk powder or the liquid supplements were fed, and conclusions drawn from the growth during the first 5 weeks. Series II refers to the experiments in which these supplements were fed in addition to a small portion of whole wheat.

Curves showing the vitamin B ( $B_1$ ) value (and presumably that of the new factor) of whole wheat are included. It is evident that 0.2 gm. daily of whole wheat carries a small but demonstrable amount of vitamin B. Some of the animals became polyneuritic and died after about 3 weeks, but several of them maintained their weight or even made slight gains. The curve shown represents the average of results from twenty-nine animals.

It seems evident that a combination of this small amount of

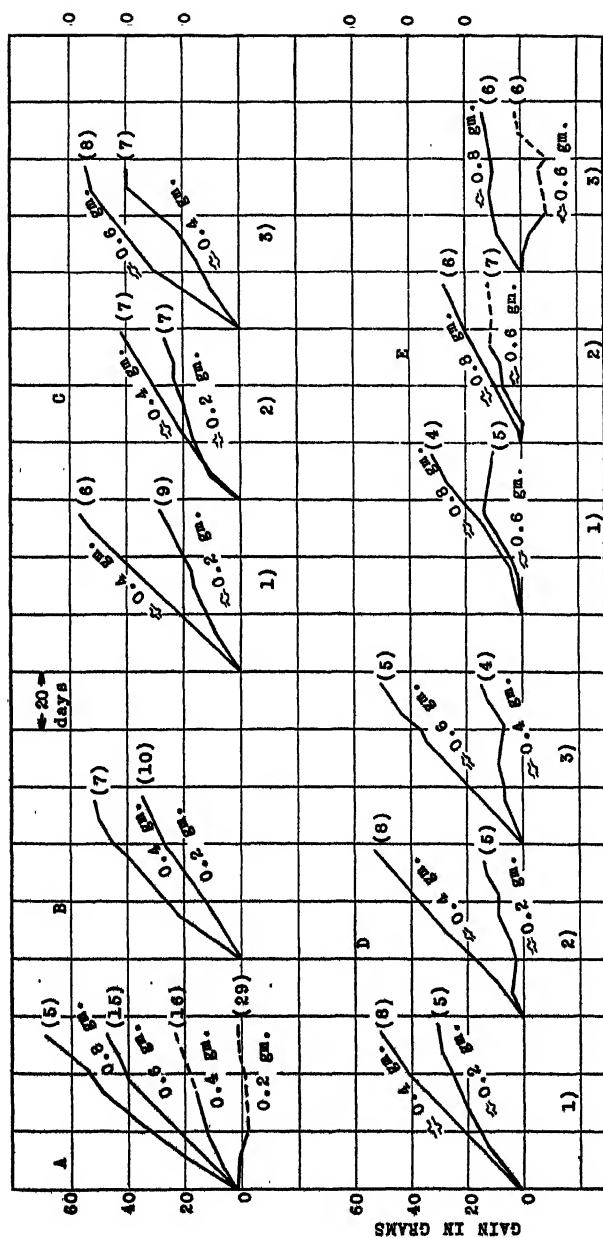


Fig. 2. Average growth curves of rats which received the Chase and Sherman vitamin B- (B<sub>12</sub>) deficient diet plus graded portions of ground whole wheat (Group A), 0.2 gm. daily of ground whole wheat plus graded portions of skim milk powder or of protein-free milk at pH 4.3, 7, or 10 respectively (Groups B, C, D, and E). Subgroups 1, 2, and 3 in each case refer to the unheated solutions, solutions heated 1 hour, and those heated 4 hours. The figure at the end of each curve indicates the number of animals of which results are included. The broken line indicates that one or more rats have died, and the curve from this date represents results of the surviving animals.

whole wheat and a portion of one of the other supplements leads to a better than additive result. In very few cases is there the flattening of the growth curves as seen in the earlier work. The results with animals receiving the solutions at pH 10 are particularly interesting. It might almost seem that the induced growth of these animals could be taken as an indication of the potency of the whole wheat as regards the new factor.

In Table I an attempt is made to compare the vitamin potency of the materials, and therefore the percentage destruction due to heat or change in hydrogen ion concentration, or a combination of these factors. Under Series I are shown conclusions drawn from a comparison of the growth attained during the first 5 weeks of the experimental period, the gain induced by skim milk powder being used as a standard. In Series II the growth is not due wholly to the skim milk powder or liquid supplement, but results from feeding such supplement plus 0.2 gm. of whole wheat daily. All of the animals, however, received the same amount of whole wheat. It was thought justifiable, therefore, to group together all supplements which, when fed in addition to the whole wheat, induced a gain of 0 to 15 gm., 15 to 30 gm., 30 to 40 gm., etc. From these groupings the conclusions shown under Series II are obtained.

For comparison are shown the results as reported in the earlier paper (8) when these same materials were tested for their vitamin G potency. It is of interest to note that under the conditions of these experiments and with the methods of testing and of evaluating results as outlined, very nearly the same degree of destruction of each factor is obtained.

#### DISCUSSION

As a result of the experiments just described it seems evident that the method of testing for the presence of vitamin B ( $B_1$ ), in rather highly purified materials especially, may need to be modified to include some source of the new factor (or factors) apparently present in whole wheat. The technique used in these experiments (feeding a small portion of the wheat in addition to the other supplements) is laborious. It was considered necessary to use this method, however, rather than to include the wheat in the basal diet, because of the high vitamin B ( $B_1$ ) content of whole wheat. Attempts are being made in this laboratory to extract the

factor by various solvents. If an extract can be prepared, very low or deficient in vitamin B and carrying the new factor, this could be included in the basal diet, as the alcoholic extract of vitamin B ( $B_1$ ) is included in the vitamin G- ( $B_2$ ) deficient diet, according to the method of Bourquin and Sherman (13).

The results show further that in the medium used and under the conditions of these experiments, the degree of destruction of vitamin B ( $B_1$ ) did not differ greatly from that of vitamin G ( $B_2$ ). It is true that the method for determination of vitamin G is rather more quantitative (under these conditions) than that of vitamin B, but when the results of both series are considered, and contrasted with those from the study of vitamin G, the difference is not great. It may be possible that the use of the terms heat-labile and heat-stable may have to be discontinued. Sherman (14) states that, "In general, vitamin B ( $B_1$ ) is less stable on heating than is vitamin G ( $B_2$ ), yet even the 'thermo-labile' vitamin B is more stable than is probably generally realized."

It is evident that both vitamins are almost completely destroyed by alkali even in the cold. The fact that when the alkalinity of the solution diminishes the vitamin G potency increases might suggest a reversible reaction in this case, with complete destruction in the case of vitamin B ( $B_1$ ). However, until more is known concerning the nature of vitamin G ( $B_2$ ), and especially in the light of the recently reported work of Stiebeling (15) who has demonstrated the multiple nature of this vitamin, any statement concerning its chemical nature is hardly justifiable.

To what an extent oxidation has been a factor in the losses incurred has not yet been determined. Although Narayanan and Drummond (16) found vitamin G ( $B_2$ ) to be rather stable to treatment with  $H_2O_2$ , it has been reported by other workers (4, 17, 18) that the manipulation processes used, in the presence of air, in attempts to extract the two vitamins, apparently led to greater destruction of vitamin G than of vitamin B. But here again the medium may be a factor in the results obtained, or the fact that more than one vitamin may be involved.

Whether or not the factor (or factors) apparently present in whole wheat is identical with the Reader (19) factor, or that of Coward *et al.* (20), Hunt and Wilder (21), Guha (22), or others, it is not yet possible to state. In experiments which are in progress

at the present time we are attempting to extract the vitamin by means of alcohol of various concentrations, or by ether. More work on all phases of the subject will be needed before any conclusions can be drawn.

#### SUMMARY

1. Experiments are described in which the stability of vitamin B ( $B_1$ ) to heat and alkalinity was studied. Determinations were made of the vitamin content of skim milk powder, protein-free milk at pH 4.3 and after it had been brought to pH 7 and to pH 10, and these solutions heated 1 or 4 hours in a water bath at  $97^\circ \pm 1^\circ$ .

2. The vitamin appeared to be completely extracted from the skim milk powder into the protein-free milk.

3. Heating 1 hour caused about 25 per cent loss at pH 4.3, 30 per cent at pH 7, and 70 to 80 per cent at pH 10.

4. Heating 4 hours caused 30 to 40 per cent loss at pH 4.3, 40 per cent at pH 7, and almost complete destruction at pH 10.

5. Holding the solution 1 week in the cold caused practically no loss at pH 4.3 or pH 7, but nearly complete loss at pH 10.

6. The results in general showed close similarity with those previously reported for vitamin G ( $B_2$ ) under the conditions of these experiments.

7. Further evidence is presented of a third factor necessary for growth of the rat. The basal diet of Sherman and Chase appears to lack this factor, and the bodily store of the animal becomes depleted in about 5 to 6 weeks. Whole red winter wheat is a good source of the vitamin, which is thought to be identical with vitamin  $B_4$ , as reported by Reader.

8. A discussion of assay to include this new factor is included.

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## SPECTROPHOTOMETRIC STUDIES

### I. SPECTROPHOTOMETRIC CONSTANTS FOR COMMON HEMOGLOBIN DERIVATIVES IN HUMAN, DOG, AND RABBIT BLOOD

BY DAVID L. DRABKIN AND J. HAROLD AUSTIN

*(From the Department of Physiological Chemistry and the John Herr Musser  
Department of Research Medicine, School of Medicine, University of  
Pennsylvania, Philadelphia)*

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Under proper conditions the spectrophotometer is of great value for comparing the concentration of certain hemoglobin derivatives in two solutions. For oxyhemoglobin we attain higher precision of measurement with the spectrophotometer than by gasometric technique. For absolute measurement of the concentration of pigment the spectrophotometric constant,  $A$ , is required for each derivative at appropriate wave-lengths. Although many papers (1-13) have dealt with the spectrophotometry of hemoglobin and its derivatives, technical objections may be raised against some of the earlier work and no studies have been made in which all of the derivatives we have examined have been prepared simultaneously from the same blood under the same conditions.

As a check upon the spectrophotometric technique it is desirable to have a reproducible standard available for measurement. Pure hemoglobin, 100 per cent of which can combine reversibly with oxygen, cannot be prepared consistently, so that the blood pigment itself is not suitable for this purpose. We have, therefore, used copper sulfate to test and standardize our spectrophotometric technique. Such standards have been used in other work (14), but not by those studying hemoglobin or other biological pigments.

The present paper includes the following: (a) a simple, reproducible spectrophotometric standard; (b) the absorption constants ( $A$  values) and  $\epsilon$  ratios for oxyhemoglobin, carbon monoxide hemoglobin, methemoglobin, acid hematin, and cyanhemoglobin



(the constants of acid hematin and methemoglobin have not received sufficient study heretofore, while cyanhemoglobin has not been subjected to spectrophotometric analysis); (c) the influence of solvent and dilution; (d) the analysis of mixtures of pigments, such as of oxyhemoglobin and methemoglobin; (e) the estimation of the errors of these determinations when carried out under controlled conditions.

### *Methods*

Blood from vein or heart was drawn onto powdered potassium oxalate to a final concentration of approximately 0.2 per cent. After oxygenation by rotation in a stoppered flask the blood was sampled for dilutions suitable to spectrophotometric measurements and for oxygen content, especial care being observed in the mixing of cells and plasma at time of sampling.

*Oxyhemoglobin, Neutral.* (a) *In 5 mM PO<sub>4</sub>*—0.5 cc. of blood was hemolyzed in 20 cc. of water in a 50 cc. volumetric flask and after hemolysis had occurred 25 cc. of 0.01 M PO<sub>4</sub> solution (KH<sub>2</sub>PO<sub>4</sub> + Na<sub>2</sub>HPO<sub>4</sub>) of pH 7.38 were added; the volume was made up to 50 cc. with water.

(b) *In H<sub>2</sub>O*—0.5 cc. of blood was introduced into H<sub>2</sub>O in a 50 cc. volumetric flask and brought to the mark with water.

*Oxyhemoglobin, Alkaline*—0.5 cc. of blood was hemolyzed in 20 cc. of water in a 50 cc. volumetric flask, and 25 cc. of double strength NH<sub>4</sub>OH (usually 0.8 N or 0.2 N) and water to the mark were added.

*CO Hemoglobin*—City gas was bubbled through water for 10 minutes to remove air from the system. The gas after passing through water was then bubbled for 5 minutes through about 20 cc. of the ammoniacal oxyhemoglobin prepared as above, foaming being controlled by touching the foam as it formed with a fine glass rod on the tip of which was a film of caprylic alcohol. A number of determinations were made for comparison, with pure CO prepared by the formaldehyde-sulfuric acid method.

*Cyanhemoglobin*—0.5 cc. of blood was hemolyzed in 20 cc. of water in a 50 cc. volumetric flask; 1 drop of 10 per cent aqueous solution of K<sub>3</sub>Fe(CN)<sub>6</sub> was added; after standing 10 minutes 2.5 cc. of 0.1 per cent aqueous solution of KCN were added and the solution diluted to 50 cc. with water.

*Methemoglobin, Neutral*—0.5 cc. of blood was hemolyzed in 20 cc. of water in a 50 cc. volumetric flask; 1 drop of 10 per cent aqueous solution of  $K_3Fe(CN)_6$  was added and the solution diluted to 50 cc. with water.

*Methemoglobin, Alkaline*—0.5 cc. of blood was hemolyzed in 20 cc. of water in a 50 cc. volumetric flask; 1 drop of 10 per cent aqueous solution of  $K_3Fe(CN)_6$  was added; after 10 minutes 25 cc. of double strength  $NH_4OH$  (usually 0.8 N or 0.2 N) were added and the solution diluted to 50 cc. with water.

*Oxygen Capacity*—Oxygen capacities were determined on 1 or 2 cc. samples with the constant volume machine of Van Slyke. Hemoglobin concentration was calculated as gm. of Hb per 100 cc. = 0.746 volumes per cent of  $O_2$  capacity.

*Spectrophotometric Analysis*—A Bausch and Lomb spectrophotometer of the König-Martens type (13, 15) was used. Readings were made in a windowless dark room. The determinations were carried out at  $25^\circ \pm 1.5^\circ$ , the change in temperature during a day's run not exceeding  $1^\circ$ . Additional screens were added to the lamp housing of the instrument in order *completely to shield the eye from extraneous light beams*. Care was taken also to make readings with a *dark-adapted eye*. Both of these factors were found important in minimizing physiological errors. The Bausch and Lomb instrument is well adapted to the use of narrow spectral intervals; most of our readings were taken by limiting the field to 3  $m\mu$  (30 Å.). The setting of the wave-length drum was checked periodically by adjusting for the position of the green line,  $\lambda = 546 m\mu$ , of the Hg arc spectrum. Adjustment of the elements in the spectrophotometer so that point of matching was  $45^\circ \pm 0.5^\circ$  was made before each series of determinations.

All calculations of the transmission,  $T$ , were based upon two determinations at each wave-length, one with the cell containing the pigment in the upper beam of light and the cell containing the solvent in the lower beam, the other with the two cells in the reversed position. This method of measurement is essential (Kennedy (13)).

In spectrophotometry one is concerned with the measurement of the amount of light transmitted, thus obtaining the ratio of the intensities,  $\frac{I'}{I}$ , of the transmitted and incident light.

$$(1) \quad \frac{I'}{I} = T, \text{ transmission}$$

Since in most problems the interest is in the absorption and not the transmission by solutions,  $T$  is obtained for the calculation of  $\epsilon$ , the *extinction coefficient*. The working form of the Bunsen-Roscoe (16) equation for the extinction coefficient is

$$(2) \quad \epsilon = - \frac{\log_{10} \frac{I'}{I}}{d}$$

where  $d$  is the depth or thickness of the absorbing medium (or depth of cell).  $d$  may be taken as unity, provided that the values one obtains are defined as to depth of cell, concentration, and wavelength, and the formula may be written

$$(3) \quad \epsilon = - \log_{10} T$$

The *molal extinction coefficient*,  $E$ , is defined as  $\epsilon$  obtained when the concentration of the solution is 1 mol per liter. According to the original formulation of Bunsen and Roscoe (16), the *concentration*,  $c$ , and  $\epsilon$  are proportional, and

$$(4) \quad \frac{c}{\epsilon} = A$$

the so called absorption relationship or *absorption constant*. It follows that, if the constant  $A$  is known by previous determination, the concentration may be determined spectrophotometrically from

$$(5) \quad c = A \cdot \epsilon$$

Ideally a solution of concentration 1 in a 10 cm. cell has the same absorption ( $\epsilon$  value) as a solution of concentration 10 in a 1 cm. cell. In our work concentrations are expressed in terms of gm. per cc., while the depth of cell is 1 cm., except where indicated. Absorption due to reagents used was found insignificant.

In polarization photometers of the König-Martens type, two polarizing elements are so arranged that the emergent beams of light are polarized at right angles to each other, so that match of the two halves of the field occurs at approximately 45°, while

complete extinction in one-half of the field is at exactly  $0^\circ$ , in the other half at exactly  $90^\circ$ . Thus, when an absorbing solution is being studied, two readings (in angular degrees) are obtained,  $\theta$ , the smaller angle (match between  $0-45^\circ$ ), and  $\theta'$ , the larger angle (match between  $45-90^\circ$ ) with the two cells in the reversed position. When the conditions of alignment are optimum, the sum of these readings is  $90^\circ \pm 1^\circ$ , while the angle  $\theta$  approximates  $90^\circ - \theta'$ . From the tangent law of photometers of this type, the following formula for calculating transmission has been developed.

$$(6) \quad T = \tan \theta \cdot \cot \theta'$$

It follows that

$$(7) \quad T = \tan \theta \cdot \tan (90^\circ - \theta')$$

and hence

$$(8) \quad T = \tan^2 \frac{1}{2} (\theta + (90^\circ - \theta'))$$

Formula 8 has not been used heretofore. When a slide-rule or logarithms are employed, identical numerical results are obtained by Formulas 7 and 8. The latter saves a great deal of time and labor, and is recommended. It is valid, however, only when angles  $\theta$  and  $(90^\circ - \theta')$  are closely approximate.

#### EXPERIMENTAL

*A Reproducible Standard*—A series of solutions of two preparations of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 2 N  $\text{NH}_4\text{OH}$ , varying from M/20 to M/320 of  $\text{CuSO}_4$ , were read in 1 cm. and 2 cm. cells and  $E$  determined. The Lambert-Beer law (17) held when the readings of  $\frac{1}{2}(\theta + (90^\circ - \theta'))$  were less than an angle of  $25^\circ$ . Greater variability and a systematic tendency to high results were observed when  $\frac{1}{2}(\theta + (90^\circ - \theta'))$  was greater than  $25^\circ$ , although at certain wave-lengths excellent agreement with the law held even for dilutions of M/320. All our further studies were made therefore so far as possible upon solutions sufficiently concentrated to yield values of  $\frac{1}{2}(\theta + (90^\circ - \theta'))$  not greater than  $25^\circ$  (18). Table I shows the  $E$  values obtained for the  $\text{CuSO}_4$  solutions at various wave-lengths with the strength of solutions read for each value reported. The means of the  $E$  values obtained from readings under  $24.5^\circ$

are given, since they possess the greater validity. The maximum deviation of the thirty-eight individual determinations under  $24.5^{\circ}$  at wave-lengths 510 to 600  $m\mu$  is  $\pm 1.7$  per cent. The maximum

TABLE I  
*E* Values per Cm. of Cell Depth for Solutions of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

$\lambda$ $m\mu$	Values of $\frac{E}{2}$							Mean value of $E$ derived from all readings <sup>138</sup> than $24.5^{\circ}$
	$m/20$ 1 cm.	$m/40$ 2 cm.	$m/40$ 1 cm.	$m/40^*$ 1 cm.	$m/80$ 1 cm.	$m/100$ 1 cm.	$m/320$ 1 cm.	
700	19.4	19.5	19.9	19.6	21.3	20.0	17.6	39.2
690	20.6	21.5	20.0	20.5	21.8	22.3	24.3	41.3
680	21.8	22.4	22.6	22.9	23.9	23.6		44.9
670	23.2	24.8	23.7	23.8	24.5	25.3	25.3	47.8
660	24.7	25.9	24.6	24.6	26.3	26.9		49.9
650	26.6	26.5	25.9	25.8	26.8	27.4	28.1	52.4
640	27.7	27.9	27.1	26.7	28.2	30.0		55.3
630	28.5	29.4	27.3	27.5	28.5	30.1	29.4	56.5
620	29.4	29.4	27.9	28.0	29.0	31.2		57.5
610	28.5	28.7	28.1	28.2	29.2	30.0	28.4	57.1
600	28.5	27.8	27.6	27.8	28.5	29.6		56.1
590	27.0	27.2	26.6	26.9	27.5	27.7	27.8	54.1
580	25.7	25.9	25.6	25.3	26.8	27.2		51.3
570	24.1	24.4	24.1	23.8	25.4	25.3	25.0	48.2
560	22.0	21.6	22.0	21.8	23.1	22.4		43.7
550	19.3	19.4	19.4	19.5	19.9	19.5	18.2	38.8
540	16.8	16.9	16.8	16.7	17.7	17.9		33.6
530	14.0	14.2	14.2	14.2	15.3	13.8	14.1	28.3
520	11.3	11.5	11.3	11.2	12.0	12.2		22.8
510	9.0	9.1	9.4	9.5	9.4	9.0	8.6	18.1
500	6.6	6.9	7.4	7.7	7.3	6.6		13.8
490	4.9	5.0	4.9	4.8	5.3	5.0		
480	3.2	3.0	2.9	3.0	3.2	3.4		

The values to the left of the heavy line were derived from readings of less than  $24.5^{\circ}$ ; values to the right and below were derived from readings of more than  $24.5^{\circ}$ .

\* A similar solution made from another preparation of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ .

deviation of the forty-four individual readings under  $24.5^{\circ}$  at wave-lengths 610 to 700  $m\mu$  is  $\pm 4.1$  per cent. The relationship of wave-length to the error is to be expected in view of the relative sensitivity of the eye to light of different wave-lengths (19). Thus,

in the green region of the spectrum,  $\lambda = 530 m\mu$  to  $\lambda = 550 m\mu$ , to which the eye is most sensitive the maximum deviation of the twelve readings is only  $\pm 0.5$  per cent.

*Absorption Constants of Various Hemoglobin Derivatives*

In Table II are given the ratios of  $\epsilon$  values at two wave-lengths for cyanhemoglobin and three wave-lengths for CO hemoglobin against a third wave-length for cyanhemoglobin as determined in thirty-four specimens of blood from the three species. The stand-

TABLE II  
*Ratios of  $\epsilon$  for Cyanhemoglobin and CO Hemoglobin of Same Blood at Different Wave-Lengths*

	Human, 11 specimens			Rabbit, 9 specimens			Dog, 14 specimens		
	Range	Mean	$\sqrt{\frac{\sum d^2}{n-1}}$	Range	Mean	$\sqrt{\frac{\sum d^2}{n-1}}$	Range	Mean	$\sqrt{\frac{\sum d^2}{n-1}}$
$\epsilon$ HbCN $\lambda = 551$	0.919	0.961	0.017	0.949	0.974	0.014	0.952	0.967	0.034
$\epsilon$ HbCN $\lambda = 545$	0.980			0.995			0.981		
$\epsilon$ HbCN $\lambda = 540$	0.988	0.999	0.009	0.983	1.000	0.014	0.977	0.995	0.030
$\epsilon$ HbCN $\lambda = 545$	1.013			1.028			1.006		
$\epsilon$ HbCO $\lambda = 569$	1.199	1.247	0.027	1.212	1.244	0.025	1.142	1.268	0.040
$\epsilon$ HbCN $\lambda = 545$	1.274			1.285			1.302		
$\epsilon$ HbCO $\lambda = 558$	0.979	1.024	0.027	0.956	0.994	0.021	0.981	1.047	0.026
$\epsilon$ HbCN $\lambda = 545$	1.067			1.028			1.080		
$\epsilon$ HbCO $\lambda = 539$	1.200	1.251	0.025	1.208	1.255	0.028	1.152	1.274	0.044
$\epsilon$ HbCN $\lambda = 545$	1.282			1.288			1.323		

$\lambda$  is expressed in  $m\mu$ .

ard deviation of the individual ratios from their mean is in man and rabbit about 1.4 per cent for the same hemoglobin derivative and 2.0 per cent when the two pigments are compared; for the dog the standard deviations are higher, about 3.0 per cent.

In Table III are given the range and average for  $A$  values of cyanhemoglobin, CO hemoglobin in ammoniacal solution, oxyhemoglobin in neutral and ammoniacal solutions, methemoglobin in neutral and ammoniacal solutions, and acid hematin in blood from human subjects, rabbits, and dogs.

There is no significant difference between species except pos-

	$\lambda$ $m\mu$	Human			Rabbit			Dog			General average $A \times 10^4$
		No. of spect- mens	Range $A \times 10^4$	Average $A \times 10^4$	No. of spect- mens	Range $A \times 10^4$	Average $A \times 10^4$	No. of spect- mens	Range $A \times 10^4$	Average $A \times 10^4$	
HbCN	620				11	84 - 224		7	151 - 238		43
	595				11	38 - 47	42.5	7	40 - 47	43.0	
	551	11	14.8 - 15.8	15.10	9	14.7 - 15.3	14.90	14	14.7 - 15.2	15.01	15.0
	545	11	14.3 - 14.7	14.50	9	14.3 - 14.8	14.50	14	13.9 - 14.8	14.50	14.5
	540	11	14.3 - 14.7	14.51	9	14.3 - 14.8	14.50	14	14.4 - 14.9	14.57	14.5
	510				11	19.8 - 22.6	21.4	7	20.9 - 22.1	21.1	21.3
HbCO in 0.1-0.4 N NH <sub>4</sub> OH	569	11	11.4 - 12.1	11.64	11	11.3 - 12.0	11.66	7	11.1 - 12.7	11.44	11.6
	558	11	13.6 - 14.8	14.16	11	14.1 - 15.2	14.59	7	13.4 - 14.8	13.85	
	539	11	11.3 - 12.1	11.59	11	11.3 - 12.0	11.56	7	11.0 - 12.6	11.39	11.5
HbO <sub>2</sub> in H <sub>2</sub> O	630	3	152 - 206					9	119 - 237		
	575	3	10.4 - 10.8	10.5				9	10.2 - 10.8	10.6	
	560	3	17.1 - 17.8	17.4				9	17.2 - 18.3	17.8	
	540	3	10.8 - 11.3	11.0				9	10.5 - 11.3	11.0	
	510	3	27 - 27	27				9	25 - 31	29	
	560:575	3	1.64 - 1.65	1.65				9	1.59 - 1.72	1.67	
	560:540	3	1.57 - 1.58	1.58				9	1.55 - 1.67	1.62	
HbO <sub>2</sub> in 5 mm PO <sub>4</sub> , pH 7.4	630	6	93 - 252		9	152 - 236		7	102 - 263		
	575	6	10.5 - 11.2	10.6	9	10.4 - 11.2	10.7	7	10.1 - 10.8	10.5	
	560	6	17.3 - 18.3	17.8	9	17.7 - 19.1	18.3	7	16.5 - 17.9	17.5	
	540	6	10.9 - 11.2	11.1	9	10.9 - 11.4	11.3	7	10.2 - 11.2	10.8	
	510	6	25 - 30	29	9	28 - 32	30	7	26 - 30	28	
	560:575	6	1.60 - 1.74	1.65	9	1.68 - 1.80	1.72	7	1.58 - 1.71	1.66	
	560:540	6	1.57 - 1.68	1.60	9	1.60 - 1.68	1.63	7	1.53 - 1.66	1.62	

HbO <sub>2</sub> in 0.1 N NH <sub>4</sub> OH	630	5	186 -288		7	176 -408		4	153 -257	
	575	5	11.0 - 11.6	11.4	7	10.9 - 11.6	11.2	4	10.8 - 12.1	11.2
	560	5	18.3 - 19.8	19.0	7	19.3 - 19.9	19.7	4	18.0 - 18.9	18.6
	540	5	11.3 - 12.2	11.8	7	11.6 - 12.2	12.0	4	11.3 - 12.4	11.7
	510	5	31 - 33	32	7	28 - 33	32	4	29 - 31	30
	560:575	5	1.64 - 1.71	1.67	7	1.72 - 1.83	1.76	4	1.55 - 1.73	1.66
	560:540	5	1.56 - 1.62	1.60	7	1.61 - 1.71	1.64	4	1.52 - 1.66	1.59
	630							4	212 -367	
	575							4	11.4 - 11.9	11.6
	560							4	18.4 - 19.4	19.0
HbO <sub>2</sub> in 0.4 N NH <sub>4</sub> OH	540							4	11.8 - 12.4	12.0
	510							4	29 - 32	31
	560:575							4	1.61 - 1.66	1.64
	560:540							4	1.56 - 1.60	1.58
	660							1	125	
	630	1	39		2	95 -119		5	36 - 39	37
	600				2	38 - 41				
	575	1	31.2		2	31.4 - 31.9		5	34 - 36	35
	560	1	31.3		2	28.4 - 31.4		5	32 - 34	33
	540	1	22.0		2	22.0 - 22.4		5	22 - 24	23
Mthb in H <sub>2</sub> O	510	1	17.8		2	18.1 - 18.8		5	17 - 18	17.3
	560:575	1	1.00		2	0.89 - 1.00		5	0.83 - 0.98	0.95
	560:540	1	1.42		2	1.29 - 1.40		5	1.39 - 1.48	1.43



	$\lambda$ $m\mu$	Human			Rabbit			Dog			General average $A \times 10^4$
		No. of speci- mens	Range $A \times 10^4$	Average $A \times 10^4$	No. of speci- mens	Range $A \times 10^4$	Average $A \times 10^4$	No. of speci- mens	Range $A \times 10^4$	Average $A \times 10^4$	
M <sub>t</sub> Hb in 0.1 N NH <sub>4</sub> OH	660				1	134		2	129, 121*		
	630	1	87		2	53 -54		2	48, 48*		
	620	1	60								
	610	1	36		2	35 -40					
	600	1	27		2	30 -35					
	590	1	23.3								
	580	1	19.0		2	23 -28		2	28, 29*		
	575	1	18.7		2	23 -25		2	25, 26*		
	560	1	20.0		2	20.7 -20.9		2	21.4, 22.2*		
	540	1	16.4		2	21.2 -21.5		2	21.0, 22.0*		
	510	1	23.4								
	490				2	0.90-1.00		2	0.89, 0.89*		
	560:575 560:540	1 1	1.07 1.22		2	1.13-1.20		2	1.16, 1.17*		
Acid hematin	660	1	23					7†	25.2-26.0	25.4	
	630	1	29					7†	32.2-34.3	33.0	
	555	1	13.3					7†	14.2-14.7	14.4	
	550	1	13.1					7†	13.8-14.2	14.1	
	529	1	11.8					7†	12.2-12.7	12.5	
	510	1	11.6					7†	12.1-12.5	12.3	

\* One specimen in 0.4 N NH<sub>4</sub>OH.

† The oxygen capacities used to determine these values were obtained at a different time with a somewhat different technique from that used for all the other values in this table and the spectrophotometric measurements were made on 1:1000 dilutions in a 10 cm. cell, thus also differing from the other measurements reported in this table.

sibly at  $\lambda = 558 m\mu$  in CO hemoglobin and at  $\lambda = 560 m\mu$  in oxyhemoglobin, and even in these instances the existence of a significant difference is not demonstrated unmistakably by the figures. Although the prism used in our instrument is one of high dispersion ( $n_D = 1.72$ ), we could not demonstrate differences in the position of the maxima and minima of absorption in carboxyhemoglobin of the different species, as Barcroft (20) has done using an instrument with a diffraction grating (much higher dispersion).

### *Quantitative Estimation of Two Pigments in a Mixture*

One of the uses of spectrophotometry, first suggested by the pioneer Vierordt (21), is the measurement of two or more substances in a single solution. For distinguishing oxyhemoglobin and methemoglobin it will be noted that the ratio of  $A$  values at  $\lambda 560:\lambda 575 m\mu$  is superior to their ratio at  $\lambda 560:\lambda 540 m\mu$ . In a mixture of two hemoglobin derivatives such as methemoglobin and oxyhemoglobin, both of which can be converted into a common pigment such as cyanhemoglobin for the determination of total hemoglobin present, the relative quantity of each pigment may be estimated by a reading of  $\epsilon$  for the unaltered mixture at any one wave-length at which  $A$  values are known for the separate pigments by utilizing the following relation.

$$(9) \quad \frac{c_1}{c_t} = \frac{1 - \frac{A_2}{A_1}}{1 - \frac{A_2}{A_1}}$$

where  $c_t$  = concentration of total pigment, the sum of  $c_1$  and  $c_2$

$A_1$  = established  $A$  value for Pigment 1

$A_2$  = " " " " " 2

$A_t$  = observed  $A$  value for mixture =  $\frac{c_t}{c_t}$

This formula assumes that the total hemoglobin is the sum of two parts and that the extinction coefficient of the mixture is the sum

of the extinction coefficients of the parts. Thus

$$\epsilon_t = \epsilon_1 + \epsilon_2$$

$$\epsilon = \frac{c}{A}$$

$$\frac{c_t}{A_t} = \frac{c_1}{A_1} + \frac{c_2 - c_1}{A_2}, \text{ hence}$$

$$\frac{c_1(A_1 - A_2)}{A_1} = \frac{c_1(A_1 - A_2)}{A_t}$$

From this equation Formula 9 is derived by rearrangement. By use of Formula 9 at several wave-lengths the calculated composition of the mixture with each wave-length may be compared and if in appropriate agreement may be averaged. We have tested the application of the formula by placing two 1 cm. cells in apposition in the spectrophotometer, one containing oxyhemoglobin, the other methemoglobin. Using the ratios  $\frac{\text{oxyhemoglobin}}{\text{methemoglobin}} = \frac{1}{2}$  and  $\frac{2}{1}$ , we have measured the proportion by Formula 9 with a 10 per cent error. The use of this equation rather than Vierordt's original equation (21) for determining two pigments from the  $\epsilon$  values of the mixture at two wave-lengths has the advantage of permitting greater weight to be given to measurements at wave-lengths where there is greater difference in  $A$  values and of allowing comparison at any number of wave-lengths. Furthermore, since the total pigment is determined independently, all of the data on the mixed pigment are utilized for determining the ratio of the two pigments, whereas in Vierordt's equation a part of the data on the mixed pigment is required in effect for measuring total pigment. Butterfield and Peabody (22) have used for this purpose an equation similar in general form to Vierordt's but with a different expression for the absorption constant, the latter not being comparable with our  $A$  values which are the absorption constants commonly employed.

*Rate of Conversion of Oxyhemoglobin to a Hemoglobin Derivative on Standing after Dilution*

Fig. 1 shows the rate of fall of the values for  $\frac{1}{A}$  (decrease in absorption at  $\lambda$ ) in a solution of blood in 5 mm  $\text{PO}_4$ , pH = 7.4,

or in 0.1 N  $\text{NH}_4\text{OH}$  when the blood is diluted 1:100 and 1:1000. It will be noted that while values approximately constant are obtained for several hours in 1:100 dilution, the fall in  $\frac{1}{A}$  values

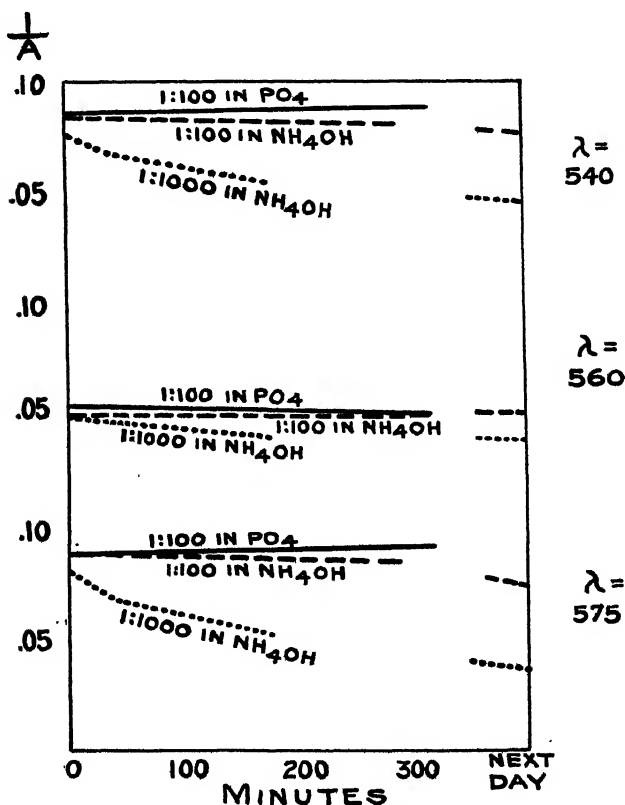


FIG. 1. Showing fall of extinction coefficient, expressed as  $\frac{1}{A}$ , with time in 1:100 or 1:1000 dilution of blood in neutral and ammoniacal solutions as measured at three wave-lengths.

in 1:1000 ammoniacal dilution is rapid enough to disturb quantitative measurements. This extraordinary instability of oxyhemoglobin in very dilute solutions has not been emphasized in the literature. Usually 10 cm. cells are supplied with our type of spectrophotometer. These cells require the use of 1:1000 dilution

for satisfactory reading of blood and therefore should not be used for hemoglobin studies. We have used specially made 1 cm. cells, thus permitting us to work with 1:100 dilution of blood.

#### DISCUSSION

Our  $A$  values for oxyhemoglobin and carboxyhemoglobin obtained upon human blood diluted in 0.1  $N$   $NH_3$  are very similar to those obtained by Kennedy (13), working under conditions closely approximating ours. The  $A$  values upon the other species differ from human blood but slightly, perhaps not significantly. At present, therefore, it remains an open question whether one should use the constants interchangeably from species to species. Contrary to Hári's (10) conclusion, solvents (water, phosphate, 0.1  $N$  and 0.4  $N$   $NH_3$ ) have a small but definite influence upon the  $A$  values.

Our  $A$  values for acid hematin differ significantly from those obtained by Newcomer (12), who used an instrument having a relatively narrow dispersion in the visible region of the spectrum.

It is to be pointed out that, once the proper technique has been mastered, spectrophotometry is not a tedious method. It is possible in a day's run to determine accurately, in the forms of oxyhemoglobin, carboxyhemoglobin, methemoglobin, and cyanhemoglobin, the hemoglobin of four or five blood specimens.

#### SUMMARY

A simple, reproducible, and adequate inorganic standard is suggested in order to standardize the spectrophotometric technique.

Values of the absorption constant,  $A$ , for various hemoglobin derivatives in man, rabbit, and dog are presented.

A method for the quantitative spectrophotometric estimation of a mixture of two pigments whose  $A$  values are known is given.

The danger of excessive dilution of blood for spectrophotometric measurement is demonstrated.

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# PHYTOCHEMICAL REDUCTION OF 1-HYDROXY-2-OXO-HEPTANE (HEPTANOL-1-ONE-2)

By P. A. LEVENE AND A. WALTI

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

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In the work on the correlation of the configurations of optically active  $\alpha$ -hydroxy acids and of secondary carbinols, the corresponding glycols were frequently made use of. When the glycols were needed as reference substances, they were prepared by the reduction of the corresponding  $\alpha$ -hydroxy aldehydes, which, in their turn, had been prepared by the ozonization of the unsaturated secondary carbinols. When larger quantities of the optically active glycols were required for the purpose of the preparation of secondary carbinols, the phytochemical reduction of the corresponding  $\alpha$ -hydroxy ketones was resorted to. In connection with the work on the configurational relationship of  $\alpha$ -hydroxyheptanoic acid to other  $\alpha$ -hydroxy acids, the optically active heptanediol-1,2 was prepared by both methods. The one obtained by the reduction of the aldehyde has been previously described.<sup>1</sup> The phytochemical method is described in the present publication inasmuch as the procedure used in the case of the lower  $\alpha$ -hydroxy ketones had to be modified in some details for the higher homologues because of their lower solubility in water.

## EXPERIMENTAL

*dl*-1-Chloro-2-Hydroxyheptane—This substance was obtained on condensation of equivalents of chloroacetaldehyde and amyl magnesium bromide. The chloroacetaldehyde was freshly prepared from 450 gm. (3 mols) of chloroacetal by slowly distilling it over 300 gm. of anhydrous oxalic acid. The distillate was taken up in dry ether and shaken for 3 hours with pulverized calcium chloride, fil-

<sup>1</sup> Levene, P. A., and Walti, A., *J. Biol. Chem.*, **94**, 593 (1931).



## 736 Reduction of 1-Hydroxy-2-Oxo-Heptane

tered, and the filtrate dropped into a well cooled ether solution of 3 mols of amyl magnesium bromide. After all the chloroacetaldehyde had been added, the reaction mixture was immediately decomposed by pouring it onto ice and acidifying with hydrochloric acid. The ethereal part was removed and the aqueous part twice extracted with liberal quantities of ether. The united ether solution was washed successively with water, sodium carbonate, and water until neutral, and dried overnight over sodium sulfate. After refractionation the chlorohydrin distilled at  $93^{\circ}$  at 13 mm. and had the following composition.

0.1244 gm. substance:	0.1194 gm. AgCl	
	$C_7H_{15}OCl$ (150.61).	Calculated. Cl 23.54
	Found.	" 23.74

The yield varied from 30 to 45 per cent of the calculated amount.

*1-Chloro-2-Oxo-Heptane (1-Chloroheptanone-2)*—The oxidation of the chlorohydrin to the chloroketone was carried out in the following manner: 100 gm. of the heptylene chlorohydrin and 65 gm. of pulverized sodium dichromate were cooled with ice and water and mechanically stirred, and 158 cc. of a 50 per cent sulfuric acid solution added in such a manner that the temperature was kept between  $10-20^{\circ}$ . When all the acid had been added, the mixture was stirred 1 hour more at room temperature and then was diluted with water and extracted with ether. The washed and dried solution (over sodium sulfate) was fractionated. The chloroketone distilled at  $80^{\circ}$  at 13 mm. The substance had the following composition.

4.125 mg. substance:	8.545 mg. $CO_2$ and 3.240 mg. $H_2O$	
	$C_7H_{13}OCl$ (148.59).	Calculated. C 56.55, H 8.82
	Found.	" 56.49, " 8.78

The yield was about 90 per cent of the calculated amount.

*1-Hydroxy-2-Oxo-Heptane (Heptanol-1-One-2)*—This hydroxy ketone was readily obtained by warming on a steam bath the chloroketone just described with its own weight of methanol and 1.25 mols of anhydrous potassium formate until the irritant odor of the chloroketone had disappeared (overnight).

After cooling, anhydrous ether was added and the precipitated salts were removed by filtration on a Buchner funnel. The salt

was extracted with ether. On concentration of the ethereal solution a fresh precipitate of salts formed and was removed. The final concentrate was fractionated, the hydroxyheptanone distilling at 87° at 13 mm. On redistillation it distilled at 95° at 20 mm. The substance crystallized in the receiver, the cooling water having a temperature of 10°. It reduced Fehling's solution in the cold. The substance had the following composition.

4.960 mg. substance: 11.660 mg. CO<sub>2</sub> and 4.795 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>14</sub>O<sub>2</sub> (130.14). Calculated. C 64.57, H 10.84  
 Found. " 64.10, " 10.82

*Dextro-Heptanediol-1, 2*.—To an actively fermenting mixture of 1 kilo of sugar and 1 kilo of bakers' yeast and 10 liters of tap water was added drop by drop a solution of 100 gm. of heptanol-1-one-2 in 150 cc. of alcohol. *The mixture was stirred continuously.* On each of 5 successive days, about 100 gm. of fresh yeast were added. After 6 days the mixture was worked up. If a filtered sample still reduced Fehling's solution, the material was extracted with ether to recover most of the unchanged hydroxy ketone for further fermentation. The aqueous part was concentrated to a viscous liquid under reduced pressure. It was then treated with absolute alcohol and a small amount of dry ether. The precipitate was removed by filtration on a Buchner funnel and washed with alcohol-ether. The filtrates were concentrated again and the residue once more treated with absolute alcohol and an equal amount of dry ether. The filtrate was concentrated and fractionated. The main fraction of the heptylene glycol distilled at 97–98° at 1.5 mm. or at 93–94° at 1 mm. The substance had the rotation of  $\alpha_D^{25} = +2.9^\circ$ . On redistillation the middle fraction (b.p. 121–122° at 12 mm.) had a rotation of  $\alpha_D^{22} = +3.30^\circ$ . The specific rotation of the substance was

$$[\alpha]_D^{25} = \frac{+1.97^\circ \times 100}{1 \times 11.72} = +16.81^\circ \text{ (in absolute alcohol)}$$

The composition of the substance was as follows:

4.491 mg. substance: 10.460 mg. CO<sub>2</sub> and 4.780 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>14</sub>O<sub>2</sub> (132.16). Calculated. C 63.59, H 12.20  
 Found. " 63.51, " 11.91

## 738 Reduction of 1-Hydroxy-2-Oxo-Heptane

*Diphenylurethane of Dextro-Heptanediol-1,2*—The diphenylurethane of this glycol was prepared in the usual manner. It was twice recrystallized from dilute alcohol. It melted at 111–112° and had the following composition.

0.1000 gm. substance: 5.63 cc. 0.1 N HCl (Kjeldahl)  
 $C_{21}H_{26}O_4N_2$  (370.32). Calculated. N 7.57  
 Found. " 7.88

The urethane had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+ 1.45^\circ \times 100}{1 \times 11.94} = + 12.14^\circ \text{ (in absolute alcohol)}$$

*Dextro-1-Bromo-2-Hydroxyheptane*—This active heptylene bromohydrin was prepared in a manner similar to that described for the levo-propylene bromohydrin from levo-propylene glycol.<sup>2</sup> 24 gm. of dry hydrogen bromide were passed into 35 gm. of dextro-heptylene glycol ( $\alpha_D = +2.85^\circ$ ) which was kept in an ice-water mixture. The reaction mixture was allowed to stand overnight. Chloroform and ice were then added and the product worked up as mentioned for levo-propylene bromohydrin. On distillation a main fraction (30 gm.) was obtained which distilled at 102° at 14 mm. The second fraction (10 gm.) distilled at 103–117° at 14 mm. and was less pure. On redistillation the first fraction distilled at 75° at 1.5 mm. Its composition was as follows:

0.1307 gm. substance: 0.1236 gm. AgBr  
 $C_7H_{15}OBr$  (195.07). Calculated. Br 40.97  
 Found. " 40.24

The rotation of the substance was  $\alpha_D^{22} = +2.0^\circ$  (without solvent).

<sup>2</sup> Levene, P. A., and Walti, A., *J. Biol. Chem.*, **68**, 415 (1926).

## PREPARATION OF EXTRACTS OF THE ANTERIOR PITUITARY-LIKE SUBSTANCE OF URINE OF PREGNANCY

BY PHILIP A. KATZMAN AND EDWARD A. DOISY

(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis)

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During the past few years a number of investigators have described preparations which stimulate the gonads of immature animals to precocious maturity. The anterior lobe of the hypophysis (Evans, 1924; Zondek and Aschheim, 1927; Smith and Engle, 1927; Putnam, Teel, and Benedict, 1928; Claus, 1931), urine of pregnant women (Zondek and Aschheim, 1927, 1928; Biedl, 1928; Evans and Simpson, 1929; Reiss and Haurowitz, 1929; Wallen-Lawrence and Van Dyke, 1931; Claus, 1931; Dickens, 1930), and human placenta (Zondek, 1929, *a*; Philipp, 1929, 1930; Wiesner and Patel, 1929; Wiesner, 1930; Collip, 1930) have been used as sources of the gonad-stimulating substance.

Of the earlier procedures, the method which has attained the most wide-spread use is that of Zondek (1929, *b*) and Evans (1929), according to which the urine is concentrated, the active principle precipitated by the addition of alcohol, and the aqueous solution dialyzed to remove inorganic salts. Acid extracts of the gland have been used by Evans and Simpson (1928), Biedl (1928), Bellerby (1929), and Hewitt (1929), but Parkes and Hill (1930), Burns (1930), and Evans (1924) have utilized extraction with aqueous alkali. R  th, Hirsch-Hoffmann, and Wulk (1928) and Steinach and Kun (1928) have prepared neutral aqueous extracts from anterior lobe tissue. After purification of the extracts by concentration and electro-dialysis or adsorption, Biedl (1928) obtained products having a potency of 0.2 to 0.05 mg. per mouse unit. Riddle and Flemion (1928) have found that glycerol extracts of beef pituitary glands are more effective than homotransplants in stimulating gonad growth in doves. Wiesner and Crew (1929) have obtained active extracts from placenta with an aqueous 20 per cent solution of sulfosalicylic acid as the extracting medium.

Dickens (1930) precipitated the active principle of urine by saturating it with ammonium sulfate and purified the aqueous solution of the precipitated material by means of dialysis. The effective dose in immature mice was

0.017 mg. Further purification by means of tannic acid and  $\text{Ba}(\text{OH})_2$  resulted in a product having a potency of 0.006 mg. per mouse unit. No data were cited regarding the yield or in how many instances preparations of such activity were obtained.

Reiss and Haurowitz (1929) precipitated the active principle with uranyl acetate and extracted the precipitate with ammonium phosphate, while Fischer and Ertel (1931) used kaolin as the adsorbent and dilute ammonia for the elution of the hormone.

Claus (1931), using Armour's anterior lobe powder, published a method for separating the follicle-ripening principle from the luteinizing substance. The hormones were extracted by acid-alcohol and the final separation of the two substances made by glacial acetic acid. The follicle-ripening factor crystallized from the acetic acid in combination with sodium chloride. Inasmuch as the effective dose of these crystals was 0.026 mg. per mouse unit, it is not likely that this material is actually the pure active principle.

Fevold, Hisaw, and Leonard (1931) reported that they obtained active extracts from dried anterior lobe by means of 50 per cent aqueous pyridine. This extract was evaporated to dryness and the residue extracted with water. According to their report, the gonad stimulator alone was dissolved while the luteinizing factor remained in the insoluble fraction. Further purification was effected by precipitation with alcohol but no statement was made regarding the purity of the final product.

Brouha and Simonnet (1927) are the only investigators besides Claus who have reported that lipid extracts of beef pituitary glands are capable of causing canalization of the vagina and estrous cycles in rats 20 to 30 days old. These extracts also reestablished cycles in castrated animals, but aqueous extracts were without effect. No method of preparation was given.

In spite of the vast amount of work which has been done in the attempt to obtain purified extracts from hypophyses or urine of pregnancy, few satisfactory methods have been developed. The difficulties are due primarily to the lability of the compound and to the failure to discover a solvent which will selectively extract the compound from the numerous substances associated with it. These limitations which have prevented rapid progress in the isolation have led us to examine the possibilities of adsorption and subsequent elution of the active material.

Our experiments which have been carried on during the past 3 years have given us a considerable amount of information about the preparation and properties of the follicle-stimulating substance which may be of value to others interested in the same problem. Of course, one of the fundamental problems of the biochemist is the isolation of the active compound. For that purpose a rapid method of preparation which yields a highly potent extract con-

taining a large proportion of the original quantity of the hormone is desirable. We believe that our acetone-benzoic acid method possesses those desirable features.

### *Alcohol Precipitation Method*

Our first attempt to obtain large quantities of the ovary-stimulating substance was carried out according to the procedure of Evans (1929) and Zondek (1929, b). The urine was slightly acidified (just acid to bromothymol blue) with acetic acid and filtered. The filtered urine was then concentrated under diminished pressure at 40° to one-tenth of the original volume. To the filtered

TABLE I  
*Extracts Prepared by Alcohol Precipitation*

Preparation No.	Urine used	Mouse units obtained per liter	Solids per mouse unit
	<i>liters</i>		<i>mg.</i>
4	50	1300*	
5	125	<300*	
6	200	<2000*	
7	225	400*	3.00
8	180	3000*	0.40
C-48p	1	250	
C-54p	1	3400	
C-56p	1	2100 rat units	

\* Assayed on the basis of vaginal opening. Smears not made.

concentrate enough 95 per cent ethyl alcohol was added to produce a concentration of 75 per cent and the resulting precipitated material dissolved in the smallest possible amount of water. This precipitation with alcohol was repeated twice more, the final precipitate being dissolved in water and assayed.

While for small amounts of material this method may be satisfactory, especially if one does not desire a product of a high degree of purity, there are some very undesirable features when large quantities of urine must be handled. In the first place, the concentration of the original urine is of necessity a slow and troublesome process, inasmuch as it is difficult to prevent frothing during the later stages of the evaporation. Furthermore, we believe that

much of the activity is lost during the long period of time during which the temperature is maintained at 40°. Then, too, very large quantities of alcohol are required for the precipitation of the active substances, making the handling of the material cumbersome. For example, if a 200 liter batch of urine is concentrated to one-tenth this volume, it is necessary to use 60 liters of 95 per cent ethyl alcohol for the precipitation. Further, as shown in Table I the yield is usually not very good and the product is crude.

Preparations 4 to 8 were assayed on the basis of opening of the vagina only. Had the assay been conducted according to our later procedure these values would have been much lower. The better yield obtained in the last two preparations (C-54p, C-56p) is due undoubtedly to the fact that the time required for concentration was relatively short since only one liter of urine was used, and also that precipitation from alcohol was performed only once. Invariably, attempts to purify the material by repeated precipitation by means of alcohol have resulted in large losses of activity.

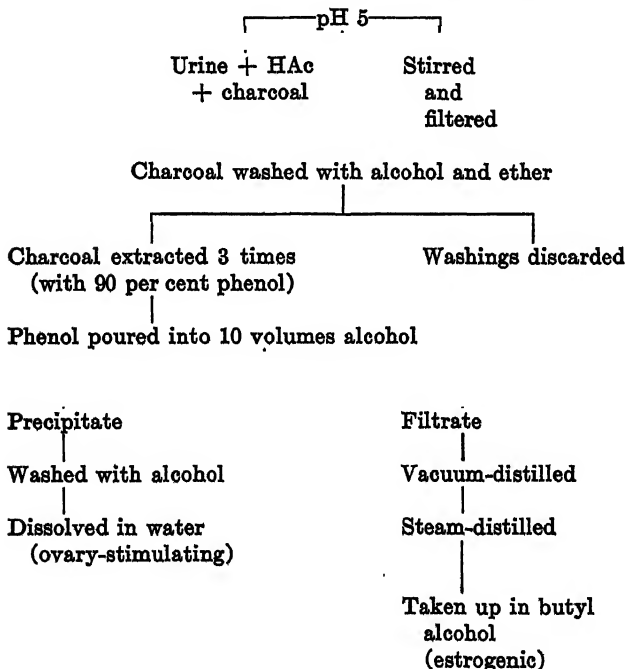
#### *Charcoal-Phenol Method*

From its behavior toward various precipitants, salting out procedures, and the reported inactivation by proteolytic enzymes, it seems likely that the active principle is protein-like in character or is associated with a protein complex. This has led us to investigate various methods used to effect protein purification, especially those used in the preparation of insulin. We have found that the method used by Dingemans (1928) and by Jensen and De Lawder (1930) to obtain crystalline insulin, when suitably modified, can be directly applied to urine to yield active extracts of a fair degree of purity. While we were conducting these experiments we were entirely unaware of Biedl's (1928) statement that he used animal charcoal and dilute phenol to prepare extracts from fresh glands. Only the mere statement without amplification or details is given.

The procedure outlined in Diagram 1, is as follows: The urine is acidified with glacial acetic acid to pH 5 (acid to methyl red-methylene blue) and filtered. To each liter of the filtered urine are added 10 gm. of activated charcoal or better still, norit, since filtration takes place more rapidly when the latter product is used.

After the charcoal-urine mixture has been stirred for half an hour or more the pH is readjusted to 5 and the charcoal is collected on a Buchner funnel by suction filtration. It is then washed once with 95 per cent ethyl alcohol, followed by a single washing with dry, peroxide-free ether, and spread to dry at room temperature. The alcohol and ether washings are discarded, as they contain

DIAGRAM 1

*Method of Preparing Ovary-Stimulating Extracts*

negligible quantities of the estrogenic hormones. The washings of the charcoal obtained from 75 gallons of urine contained between 26,000 and 55,000 rat units (Allen-Doisy) or 100 to 200 rat units per liter of urine.

The dry charcoal is mixed intimately with sufficient 90 per cent phenol to make a rather thin paste and filtered by suction. This process is repeated three times and the combined phenol extracts are poured into 10 volumes of 95 per cent ethyl alcohol. The



precipitate which contains the desired material is allowed to settle in the cold room and after syphoning off the supernatant liquid it is collected by centrifugation and washed repeatedly with alcohol. It is then taken up in water and the insoluble material removed by centrifugation. These preparations have a deep brown coloration but contain relatively little solid material.

We have studied the efficiency of norit and activated charcoal (Mallinckrodt) for the adsorption of the estrogenic and anterior pituitary-like principles in a series of twenty-one experiments. Our data indicate that under the conditions used at least 95 per cent of the former and 80 per cent of the latter are adsorbed. Of course, it must be understood that in the case of the follicular hormone accuracy of the data depends upon the preliminary assay on spayed rats before adsorption and then a subsequent assay after stirring with the adsorbent. In the case of the ovary-stimulating principle, separate samples of the urine before and after the adsorption were carefully acidified, extracted with butyl alcohol, and then assayed on immature rats and mice. Because of the lability of this principle, the factor of destruction may introduce an error in our estimate of the extent of the adsorption.

The yield and purity of the preparations by the charcoal-phenol method are of considerable interest. Of thirty-five separate preparations the highest yield, 24,000 mouse units per liter of urine, was obtained from a pregnant woman who showed symptoms of hyperpituitarism. The average yield was about 2000 mouse units per liter but many preparations gave around 3000 and some as little as 200.

Since the estrogenic substances, theelin and theelol, are quite readily soluble in strong alcohol and acetone solutions the above method renders improbable their presence in the follicle-stimulating extracts. Nevertheless, for additional assurance these extracts were assayed on adult, spayed female rats. The number of mouse units of ovary-stimulating hormone of various preparations required to produce estrus in the castrated animal varied from 200 to 1700. In most instances the amount of contamination was negligible.

As stated in a preceding paragraph the treatment of the urine with charcoal removes theelin and theelol as well as the ovary-stimulating substance. If the extraction of the charcoal by

phenol removes these substances they should be found in the alcohol-phenol solution from which the precipitated ovary-stimulating material has been removed. An investigation of this point revealed that considerable quantities of these hormones are obtained by elution with phenol. The alcohol is removed by distillation and the phenol by distilling with steam. The resulting mixture is then evaporated to dryness and the residue taken up in butyl alcohol or sodium hydroxide solution, diluted, and assayed on spayed rats in the usual way. The quantities of material which we have obtained in this manner ranged from 500 to 6500 rat units per liter of urine, the average of nine preparations being 2750.

In spite of the appreciable amounts of theelol and theelin present in the residue remaining after distillation of the alcohol and phenol it has been practically impossible to obtain these substances in pure crystalline form. The attempt toward this end consumed several months of laborious effort. When the procedures whereby theelin and theelol are obtained directly from urine (Doisy, Veler, and Thayer, 1930; Doisy and Thayer, 1931) were applied to these residues, the final product was a red tar which was sometimes interspersed with a few crystals. The separation of this mixture was exceedingly difficult. After various modifications and introduction of new steps in the procedure, theelol was obtained in pure crystalline form in two instances (50 and 20 mg.) and in one case 10 mg. of theelin were recovered. The theelol melted at 271° (uncorrected) and had an activity of 1500 rat units per mg.; the theelin melted from 235–245° (uncorrected) and had an activity of 2800 rat units per mg. This method, according to our experience, is not practical for obtaining theelin and theelol.

#### *Benzoic Acid Method*

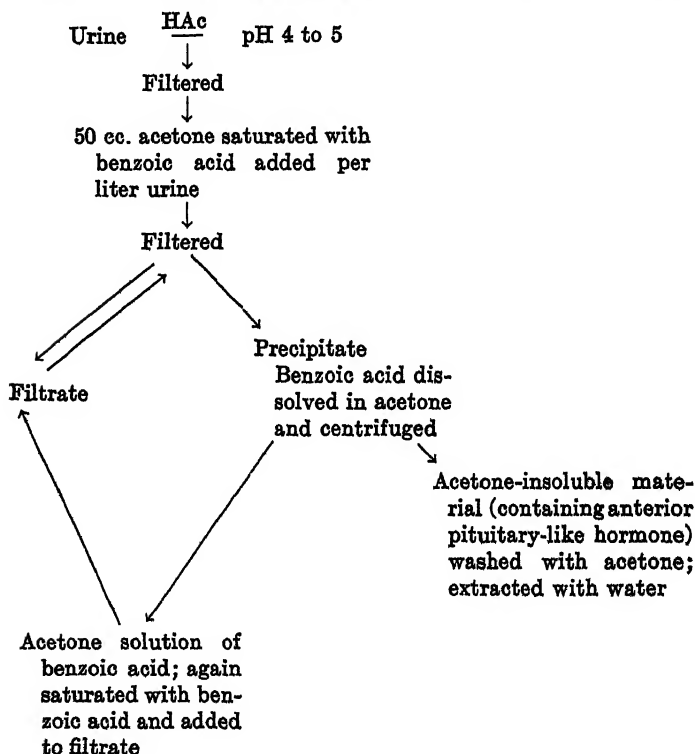
While we were preparing extracts of the ovary-stimulating factor from urine by the alcohol precipitation method, we attempted to purify these very crude preparations by adsorbing the active principle on benzoic acid precipitated in the extract. For this purpose, a saturated solution of sodium benzoate was added and the benzoic acid precipitated by the careful addition of dilute hydrochloric acid (Moloney and Findlay, 1923). The same result was attained by adding to the extract a saturated solution of benzoic acid in alcohol. In most cases this treatment removed

about 50 per cent of the activity. When the latter precipitation is carried out directly in urine, considerable quantities of the active material are carried down with the precipitate.

The essential steps of the procedure are indicated in Diagram 2. The urine which is preserved with a little  $\text{CHCl}_3$  and kept in the

DIAGRAM 2

*Preparation of Ovary-Stimulating Extract by Benzoic Acid Method*



cold room ( $1-5^\circ$ ) is made acid to methyl red-methylene blue by the addition of glacial acetic acid and filtered or clarified with a Sharples centrifuge. The urine is vigorously stirred with a mechanical stirrer while acetone saturated with benzoic acid (50 cc. per liter of urine) is added. The urine thus treated is usually allowed to remain in the cold room overnight. The precipitated

TABLE II  
*by Benzoic Acid A.*

Preparation No.	Mouse units per liter		Hormone obtained, mouse units per liter urine			Per cent recovered	Purity of extracts, mg. per mouse unit	
	Original urine	After adsorption	1st ppt.	2nd ppt.	Total		1st ppt.	2nd ppt.
C-41C		<1650	10,000				0.006	
C-44C	4,200 rat units	<850 rat units	4,200				0.008	
C-48C		<800 " "	2,350				0.019	
C-50C	<2,000 rat units		2,800				0.004	
C-51C	2,700 " "		2,000					
C-54C	>4,200	<1650	4,400			<100	0.004	
C-56C		<400 rat units	8,000				0.003	
C-59C		<2700	5,000				0.006	
C-57C		5000	1,000	500	1,500		0.013	0.016
C-58C	20,000	2000	8,000	2000	10,000	50	0.004	0.007
CB-60C	15,000	500	2,500			17	0.015	
CC-60C	15,000	2500	2,500			17	0.005	
CA-63C	8,500		6,000			70	0.006	
CB-63C	8,500		6,000			70	0.006	
CC-63C	8,500	4000	5,000			60	0.006	
C-64C		1100	1,500	4000	5,500		0.006	0.006
C-67C	8,000	3000	4,000	500	4,500	56	0.023	0.080
C-69C	8,000		5,000			63	0.012	
C-70C	8,000	<1000	1,600	550	2,150	27		
C-71C	7,000	<1000	4,250	300	4,550	65		
C-72C	11,000	<1000	1,250	2300	3,550	32		
C-73C	8,500	<1000	>4,500	550	>5,500	65		

benzoic acid is collected by suction filtration and dissolved in a volume of acetone equal to the volume originally added. The small amount of acetone-insoluble flocculent material which contains the active principle settles rapidly and is easily and quickly obtained by syphoning off most of the supernatant liquid and centrifuging the remainder. After thoroughly washing the precipitate with acetone to remove benzoic acid and occluded theelin, it is extracted three times with distilled water, being centrifuged after each extraction. The amount of water used depends, of course, upon the bulk of the precipitate. For 1 liter of urine we have used 25 cc. of water and for 100 liters, 300 to 500 cc. of water. Beginning with the filtration of the precipitated benzoic acid, the whole procedure for a batch of 100 liters of urine can be carried out in 8 to 10 hours.

An additional quantity of the hormone may be recovered by repeating the benzoic acid adsorption upon the urine filtrate obtained from the first precipitation. For this purpose the acetone solution of the benzoic acid recovered from the first precipitation is re-saturated with benzoic acid and added to the urine. The yield and purity of the preparations are shown in Table II.

The first eight preparations listed in Table II were prepared by a procedure somewhat different from that just described, in that the benzoic acid was dissolved in alcohol instead of acetone before being added to the urine. Although the yield and purity of these products were satisfactory, we had reason to suspect the destruction of the active principle by the alcohol or impurities contained in the alcohol. Therefore, we have eliminated entirely the use of this solvent by employing a saturated solution of benzoic acid in acetone. From the data presented it appears that this change has not resulted in any improvement. The concentration of the alcohol was probably too low to injure the hormone.

It will be observed that although in most instances the method gives fairly good recovery (50 to 70 per cent) of the active principle, low yields have been obtained in a few cases (Preparations CB-60C, CC-60C, C-70C, C-72C). The amount of activity remaining in the urine after adsorption indicates that especially in these cases either destruction or loss of the active principle has occurred. By means of a special extraction method we have been able to show that the acetone-insoluble fraction, after extraction with water,

contains appreciable quantities of the hormone and that this fraction is richer in the hormone when the yield by aqueous extraction is poor than when it is good. However, the additional amount of the active material which we have obtained by this means does not entirely account for the discrepancy.

The concentration of the active principle in the urine, before and after adsorption, was determined by assaying a portion of the urine after it had been extracted three times with an equal volume of butyl alcohol and once with ethyl ether to remove the estrogenic hormones. This extraction process causes a considerable reduction of the volume of the urine which must be taken into account in the assay. Since the extracted urine was not tested on spayed rats it is possible that the estrogenic hormones were not completely removed. In this event the recovery of the ovary-stimulating factor may be greater than is indicated in Table II. On the other hand, it is possible that the butyl alcohol destroys some of the anterior pituitary-like hormone. If this is true, then the values given in the first two columns of Table II are too low.

In those instances where a second precipitation was carried out the additional material recovered varied from about 10 to 300 per cent of the yield of the first precipitation. Inasmuch as the acetone solution of the first benzoic acid precipitate may be used again, this step is undoubtedly worth while.

It can readily be seen that this method is far the best of the three methods described, from the standpoint of purity of product, yield, and ease of manipulation. It is indeed gratifying that so rapid and simple a procedure yields considerable quantities of the active material in so pure a condition.

Potent extracts can also be prepared by the use of suitable organic acids other than benzoic acid. To separate samples of a urine containing 8500 mouse units per liter, benzoic, cinnamic, stearic, salicylic, and phthalic acids were added. The yields of the anterior pituitary-like principle were 60, 60, 30, 14, and <7 per cent, respectively. Cinnamic acid is apparently as effective as benzoic acid, but the difference of cost makes the latter preferable.

*Purification*

The extracts obtained by the benzoic acid method can be considerably purified merely by a single repetition of the procedure, 5 to 10 cc. of the benzoic acid-acetone solution being used for every 100 cc. of the extract. The purification effected is presented in Table III. Although the amount of hormone recovered by this procedure is not quantitative, the purity of the resulting product makes this step valuable.

TABLE III  
*Purification of Extract by Benzoic Acid Adsorption*

Preparation No.	Adsorption with benzoic acid		Per cent recovered	Total solids		Before adsorption	After adsorption
	Mouse units used	Mouse units recovered		Used	Recovered		
				mg.	mg.	mouse units per mg.	mouse units per mg.
C-41C	31,000	25,000	80	380	19	83	1250
C-58C	60,000	30,000	50	484	26.5	125	1250
C-57C <sub>1</sub>	10,000	7,000	70	286	16	40	440
C-64C <sub>2</sub>	84,000	46,000	55	1160	180	73	260
C-68C	80,000	60,000	75	1135	100	65	585

TABLE IV  
*Purification by Fractional Precipitation with Acetone*

Preparation No.	Before	After	Solids per mouse unit
	mouse units per mg.	mouse units per mg.	mg.
41 C <sub>2</sub> A <sub>1</sub>	1250	3800	0.00026
41 C <sub>2</sub> A <sub>2</sub>	1250	3000	0.00033
68 C <sub>2</sub> A <sub>3</sub>	580	3400	0.0003

The concentration of the hormone in the extracts before the purification recorded in Table III varied between 170 and 340 mouse units per cc. Apparently, if the concentration is much greater than this the adsorption is not effective. In one instance, with a preparation assaying 1650 mouse units per cc., we recovered only 10 per cent of the activity by this procedure, the remainder being unadsorbed. Such concentrated solutions, then, should either be diluted or treated with larger quantities of the benzoic

acid solution. Dilution is preferred since in the latter case the concentration of acetone would become too great.

Further purification is attained by fractional precipitation with acetone. (Acetone precipitation was used by Evans, Meyer, and Simpson (1932) to prepare dry powders from glands and urine.) From its concentrated solution the active material is precipitated when the acetone concentration is between 50 and 60 per cent. Above or below these limits the precipitated material is relatively inactive, but the 50 to 60 per cent precipitate is quite active and in some instances assayed fully 3000 mouse units per mg. (Table IV). These preparations give positive biuret and Millon's reactions. By a procedure which is as yet not adequately standardized we have increased the potency to 5000 mouse units per mg. of solid material without eliminating the biuret reaction.

### *Bioassay*

In our earlier work we attempted to use the opening of the vagina as the sole reaction for a method of assay (Table I). However, it soon became apparent that this procedure was not satisfactory because it offered no means of differentiating accidental opening from true response. Also, because such amounts of material produce no macroscopic changes of the ovaries, it is difficult to rule out the effect of minute traces of theelin and theelol.

In the assay of our preparations we have taken as the mouse unit, the minimum quantity of material which, administered subcutaneously to 19 day-old mice in six equal portions during the course of 3 days, causes opening of the vagina *and estrus* on the 22nd to 24th day of age. We have also used 21 day-old rats, in which case 1 rat unit must produce these reactions by the 27th day of age. In the control mice opening of the vagina occurs, on the average, on the 30th day and estrus on the 48th day. For the control rats these ages are 46 and 48 days, respectively. Assays of the same preparations on both immature mice and rats show that approximately 4 times as much material is required to produce these effects in the rat as in the mouse.

This serves as an adequate basis for assay if one is assured of the absence of the estrogenic hormones which are also capable of inducing the phenomena in question. For such assurance one should rely upon the adult spayed female rat in which the ovary-



stimulating factor is without effect. Such assay of some of our extracts prepared by the benzoic acid-acetone method shows that estrogenic material is either entirely lacking or present in insignificant amount. Nine different preparations have been tested on spayed rats with quantities ranging from 330 to 1675 mouse units of the ovary-stimulating principle. Cornification did not occur in any test, thus indicating the practical absence of the estrus principle. In this event there is no need of using the more difficult procedure of sacrificing the animal for examination of the ovaries.

Furthermore, we believe that it is correct to use the characteristic reaction, *i.e.* the stimulation of follicle ripening, as a measure of potency and that this reaction lends itself more easily to a quantitative interpretation.

Since it is quite certain that these extracts are free from estrogenic material, 1 unit of the anterior pituitary-like factor must stimulate the ovaries sufficiently to cause opening of the vagina and estrus. It is necessary to establish this point in order to demonstrate that our assay is based upon a definite ovarian response. At the present time Dr. Collier,<sup>1</sup> of the Department of Pathology, is making a histological study of ovaries taken from immature rats which have been treated with small amounts of the ovary-stimulating extracts. In the following statement he has briefly summed up his findings.

"A preliminary study of the effect of this material demonstrates no change qualitatively different from that seen in normal animals. There is, however, a considerable quantitative change. The earliest quantitative change is an increase of the number of atretic follicles and an increase in the development of other follicles beyond the stage found in normal animals of the same age. Some of these developing follicles matured and were followed by the formation of normal corpora lutea of ovulation at an age about one-half that of normal ovulation. The atretic follicles show a degenerative change in both granulosa cells and internal theca, particularly in the former. Some of these changes involve edema and vacuolization of the cells with some of the vacuolar contents staining with osmic acid, showing their fatty character. Such fat-laden cells have been called 'pseudolutein.' There is neither 'pseudoluteinization' nor other change in atretic follicles that has not been unmistakably found in the normal animals of different ages. On comparing the ovaries of immature rats

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<sup>1</sup> We wish to express our appreciation to Dr. Collier for his generous aid and cooperation in this work.

which have been treated with small doses of urine extracts over a short period of time with those of normal animals of the same age, the difference appears to be that the former present the appearance of a greater age with a relatively greater quantity of 'pseudoluteinized' atretic follicles."

When this work is complete we hope to publish more detailed data concerning the bioassay.

#### SUMMARY

Two new methods for the preparation of the anterior pituitary-like substance from urine of pregnant women are described. One depends upon the adsorption of the active principle by norit and elution with phenol; the other depends upon its adsorption on finely divided benzoic acid and elution by dissolving the benzoic acid in acetone.

The latter procedure is rapid, simple, and gives good yields of the hormone in fairly pure form. By this means extracts having a potency of 1250 mouse units per mg. have been obtained.

By fractional precipitation with acetone the potency has been increased to over 3000 mouse units per mg. of solids. These preparations give positive biuret and Millon's reactions.

The immature mouse and rat units, based upon opening of the vagina and estrus, are defined. Preparations made by the benzoic acid-acetone method are free from estrogenic hormones and hence there is no necessity of examining the ovaries of the test animals.

We are indebted to Miss Corinne Dewes for her assistance with the assays.

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## CYTOCHROME AND YEAST IRON

By THOMAS B. COOLIDGE

*(From the Converse Memorial Laboratory of Harvard University, Cambridge)*

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The experiments described here were begun in an attempt to titrate potentiometrically as a reversible oxidation-reduction system the pigment cytochrome, described first by MacMunn (1) and more recently and extensively by Keilin (2). This attempt was abandoned because the amount of cytochrome present in the yeast used appeared to be too small to titrate. It was found possible, however, to determine the oxidation-reduction potential indirectly, and certain other facts were discovered which appear interesting in regard to biological oxidations.

### EXPERIMENTAL

Pressed yeast (1 pound Fleischmann's bakers' cakes) was crumbled and allowed to stand overnight with an equal weight of ammonia solution (concentrated ammonia solution to water, 1:3). On centrifuging, the red-brown supernatant fluid showed a strong cytochrome "C" spectrum. The solution was neutralized to litmus with dilute sulfuric acid and ammonium sulfate was added to saturation. The precipitate of proteins so obtained was redissolved and dialyzed efficiently for several days, at the end of which it still showed a strong cytochrome C spectrum. A great many attempts to separate the material showing the spectrum from all protein by precipitating the latter with protein precipitants and absorbents in both alkaline and acid solutions were made without success. It seemed probable that the cytochrome is an integral part of some protein molecule or is unstable in the absence of protein.

The dialyzed protein solution was buffered<sup>1</sup> and titrated elec-

<sup>1</sup>The buffers used throughout these experiments were (1) mixtures of primary and secondary sodium or potassium phosphate, giving pH 7; (2)

trometrically in a gas-tight vessel through which oxygen-free nitrogen was bubbled.

Oxygen was removed by bubbling nitrogen through the solution continuously 1 hour before the titration was begun. The reagents used were approximately 0.01 N titanous chloride or alkaline

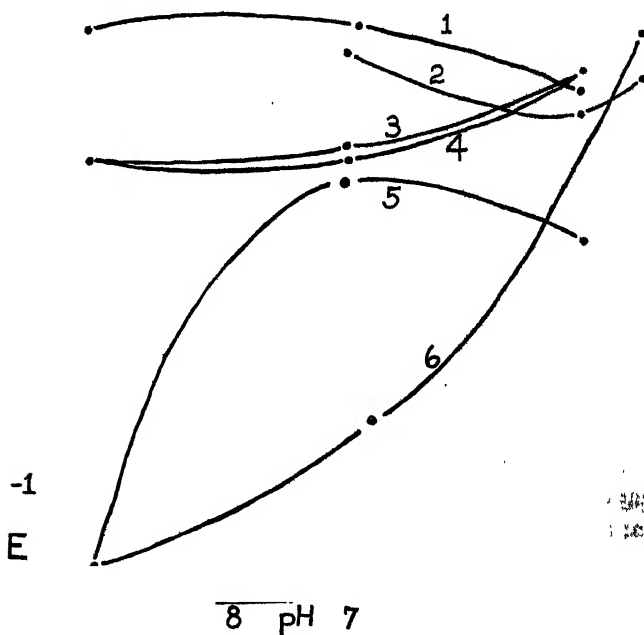


FIG. 1. Oxidation-reduction potential of cytochrome. Curve 1 shows the voltage at which the cytochrome spectrum disappears; Curve 2, cytochrome  $E_0$ ; Curve 3, mid-points of dialyzed extract curves; Curve 4, mid-points of iron-albumin mixture curves; Curve 5, mid-points of curves of protein-free iron compound prepared from dialyzed extract; Curve 6,  $E_0$ , iron pyrophosphate ( $2 \times 10^{-4}$  N).

sodium hydrosulfite solution as reductant and exactly 0.01 N  $K_2Fe(CN)_6$  solution as oxidant. All were boiled free of air in

mixtures of sodium borate and sodium tartrate giving pH 9.2; (3) mixtures of sodium hydroxide and acetic acid giving pH 4.5; (4) mixtures of citric acid and sodium hydroxide giving pH 5.0. The concentration, provided it was sufficient, appeared to have no effect on the results and is not recorded.

*vacuo* just before used and stored under oxygen-free nitrogen. Curves were obtained by plotting the volume of reagent used against the voltage.

As reductant was added no smooth curve was obtained, the voltage dropping fairly sharply from a point near the mid-point of the curves described below. On adding oxidant after reduction, curves were obtained having approximately the shape of the theoretical curves of such systems as the ferrous-ferric. The potentials of the mid-points obtained at different pH values are shown in Fig. 1. The amounts of oxidizing agent required varied from  $1 \times 10^{-6}$  to  $5 \times 10^{-6}$  equivalents with 10 cc. portions of different preparations.

The curves obtained are not entirely satisfactory for the following reasons: (1) The electrodes, which always agreed to within a millivolt both with each other and with the accepted voltages in an acid solution of quinhydrone immediately before the titration of the protein solution, practically invariably disagreed by as much as 0.1 to 0.2 volt on the parts of the curves distant from the mid-point and often by as much as 0.05 volt at the mid-point. (2) On reaching a potential somewhat above the mid-point, further addition of oxidant raised the potential momentarily but a rapid negative drift appeared quickly. Smooth curves were obtained only by titrating rapidly enough at this point to avoid the drift.

While these experiments were in progress, Keilin published a method in which the material showing the cytochrome C spectrum was extracted from yeast by boiling a suspension of plasmolyzed cells, extracting the washed precipitate with a solution containing bisulfite and hydrosulfite, precipitating most of the proteins with  $\text{SO}_2$ , filtering, and removing the cytochrome-containing material from the filtrate by aeration. On dissolving, in buffer solutions, the material so obtained, curves identical with the above were obtained on electrometric titration.

In order to see whether the material being titrated in both cases was an iron compound, iron analyses were carried out on the solutions being titrated. These were performed by titration of a solution of the wet ashed material in an atmosphere of nitrogen either electrometrically or with methylene blue as indicator, with titanous sulfate as reducing agent. The iron was oxidized to the ferric state by means of superoxol and the excess of peroxide re-

moved by boiling with iron-free ammonia solution. The method was capable of an accuracy of 2 per cent on samples containing about 0.1 mg. of iron as shown by titration of gasometrically standardized hemoglobin solutions, and is not affected by the presence of phosphate or pyrophosphate.

Four curves were obtained from different solutions of the dialyzed ammonium sulfate precipitate. In one of these cases the material after dialysis was treated with an equal volume of 95 per cent alcohol, the resulting precipitate being redissolved in buffer and titrated. The amount of oxidant used to oxidize the solution and the amount of iron present agreed in all four experiments within 20 per cent or within the limit of accuracy of the method as determined by the sharpness of the curves. The same agreement was found in titrating a preparation by Keilin's method. These solutions were about  $2 \times 10^{-5}$  N in iron.

In the course of attempts to purify the material giving the cytochrome spectrum the undialyzed precipitate brought down by ammonium sulfate was subjected, as stated above, to a variety of procedures. The material obtained was, in several cases, titrated, and it was found that although smooth curves were sometimes obtained, the amount of iron present was only one-half to one-quarter of the amount corresponding to the span of the titration curves.

The above facts indicate that, while under certain conditions an iron compound in the dialyzed extract can be titrated with  $\text{K}_2\text{Cr}_2\text{O}_7$ , there is present also a reducing effect which is marked at potentials near the mid-point potential of the iron compound. The substance causing this reducing effect may reduce amounts of oxidant which are stoichiometrically equivalent to several times the amount of iron compound present.

An attempt was next made to see how far the behavior of the above solutions could be reproduced by means of solutions of iron salts and proteins. These were prepared by shaking approximately 0.5 gm. samples of dried egg albumin with 50 cc. quantities of buffer, filtering, and adding ferric chloride solution to give an iron concentration of  $2 \times 10^{-5}$  N. These solutions on titration closely duplicated the solutions obtained from yeast. The mid-point potentials were practically identical and there was a definite though slight negative drift at points above the mid-

point potential. The same results were obtained when the iron was added to the protein solution as the complex iron pyrophosphate. The potentials obtained lie several tenths of a volt above those obtained with most iron salts (3), and indicate that a strong iron-protein complex is formed.

In order to gain further insight into the nature of the iron compound present in the solutions obtained from yeast by the method outlined above and by Keilin's method, they were treated with iron-free trichloroacetic acid, a 20 or 40 per cent solution of the acid being added slowly to the cytochrome solution until the concentration of the acid was 8 per cent in the mixture, which then stood a variable number of hours and was centrifuged, yielding a water-clear supernatant fluid showing no spectrum. Iron determinations were made on both the original solutions and on the supernatant fluid. The amount of iron recovered in the supernatant fluid varied widely in different experiments, forming from 30 to 90 per cent of that present in the original solution. On redissolving the precipitate in ammonia and adding hydrosulfite, no diminution in the spectrum was noted. This indicates that the iron compound responsible for the potentiometric curves is not cytochrome. Assuming that the iron remaining in the precipitate is all cytochrome iron, it was calculated from several experiments that the cytochrome iron is about 0.3 per cent of the total yeast iron, this total from several analyses lying between  $0.80 \times 10^{-6}$  and  $1.10 \times 10^{-6}$  equivalents of iron per gm. of pressed yeast.

In the trichloroacetic acid extracts, the iron was complexly bound, as shown by the fact that it did not give the sulfocyanide test on adding  $\text{H}_2\text{O}_2$ . It did, however, give a reaction with dipyridyl. On attempting electrometric determinations of the pH value of the solution, it was found impossible to get values which corresponded with the pH value determined colorimetrically, and the two electrodes used often disagreed with each other. The extent of the error was not quantitatively reproducible, was maximal at pH 7, and amounted at most to about 70 millivolts, the observed voltages being negative to the values actually corresponding to the pH. This behavior is observed with solutions of iron pyrophosphate, and analysis of the solutions by the method of Fiske and Subbarow (4) showed the presence of



phosphorus, not present as phosphate, to the extent of about 20 mg. per 100 cc. of fluid. It appears not to be present as pyrophosphate, as it is not precipitated by barium salts. The percentage hydrolyzed to phosphate by 1 N HCl at 100° in 7 minutes varied from 20 to 70 per cent in several preparations prepared with slight variations in method. It is hoped to investigate this compound further.

The trichloroacetic acid extracts were neutralized, buffered, and titrated electrometrically, giving curves whose mid-points at different pH values are shown in Fig. 1. These indicate that the iron is not present in the extract in the form in which it is present in the protein-containing solutions. Moreover, the protein-containing solutions have no disturbing effect on the hydrogen electrode.

These facts show that most of the iron present in these solutions is present as an iron-protein complex, the mid-point potential of which lies well above that of most iron complexes (3). This complex is much looser than the cytochrome complex, and probably at least 3 times as much iron is bound in this way as in cytochrome in the solutions studied. On removing the protein, the mid-point potential is lowered. The relation of the phosphorus-containing material to this complex is not established.

### *Cytochrome Potential*

The dialyzed extracts were now reexamined, being observed with a hand spectroscope in an attempt to correlate this voltage with the mid-point of the titration curves. It was found to lie at a point about 0.1 volt above the mid-point of the titration curve, the exact point being impossible to measure accurately because the negative drift was very marked at this point.

This difficulty was obviated by the addition of quinhydrone to the solution. This poised the voltage at a level near that at which the spectrum appeared and disappeared, and by the addition of small amounts of oxidant or reductant the exact point of disappearance could be determined to within 5 millivolts. There was no drift and the electrodes agreed to within a fraction of a millivolt. For these experiments buffered solutions of a preparation by Keilin's method were used. By measuring the volume of

the solution during this determination, then removing it from the apparatus, reducing fully with sodium hydrosulfite, and diluting until the spectrum just disappeared, it was possible to calculate the  $E_0$  potential of the pigment as follows:

Let  $x$  be the volume of the solution in the titrating vessel when the voltage at which the spectrum disappears is  $E_d$ .

Let  $y$  be the volume when the spectrum of the fully reduced solution is just made to disappear by dilution.

The concentration of reduced pigment is the same in both cases.

TABLE I  
*Cytochrome,  $E_0$  values*

Material	Buffer	pH	$x$	$y$	$E$ (against calomel cell)	$E_0$ (referred to normal hydrogen electrode)
			cc.	cc.	volt	volt
Cytochrome solution.....	Acetate	4.5	17	50	0.007	0.235
" " .....	Citrate	5.0	20	100	0.003	0.207
" " .....	Phosphate	7.0	20	200	0.068	0.256
" " .....	"	7.0	20	670	0.106	0.262
" " .....	"	7.0	20	650	0.102	0.258
" " oxy-						
genated.....	"	7.0	30	200	0.086	0.272
Yeast suspension.....	"	7.0	20	30	0.000	0.264
" " .....	"	7.0	40	73	0.000	0.251
" " .....	"	7.0	30	50	0.000	0.256

The concentration of total pigment in the first case is then  $\frac{y}{x}$  times the concentration of reduced pigment. Or

$$\frac{(\text{Reductant}) + (\text{oxidant})}{(\text{Reductant})} = \frac{y}{x} \frac{(\text{reductant})}{(\text{oxidant})}$$

$$\text{as } E = E_0 - 0.06 \log \frac{(\text{reductant})}{(\text{oxidant})}$$

$$E_0 = E_d + 0.06 \log \frac{x}{y - x}$$

The assumption that the concentration of reduced pigment in both cases is the same is subject to errors of observation and

to the fact that dilution (with water) modifies the conditions under which the spectrum is being observed.

The  $E_0$  values obtained by this method are shown in Fig. 1 and Table I.<sup>2</sup> A determination made with oxygen bubbling through the solution is included, the result agreeing with the others within the limit of error of the method. The values obtained at pH 9.2 were so far from what appeared to be the value in the absence of quinhydrone, which is unstable in alkaline solution, that they are not recorded.

The same method was applied to suspensions of yeast cells plasmolyzed with sodium chloride and suspended by phosphate buffer. The error in these determinations is considerably increased because the quinhydrone was considerably reduced, the solution was very highly colored, and the potential was drifting rapidly. The values from three consecutive experiments are given in Table I.

#### DISCUSSION

In the above experiments, the oxidation-reduction potential of cytochrome C is measured with a fair degree of accuracy and certainty. This potential is shown to be unaffected by changes in oxygen tension and to be, as far as can be judged by the above experiments, the same in the solutions as in yeast cells which have undergone no treatment except plasmolysis and suspension in buffer. This is good evidence that the cytochrome has not undergone modification during the drastic treatment used to purify it and probably indicates that cytochrome C is a hemochromogen in which the hem is tightly bound to the nitrogenous part. It is good evidence that the cytochrome does not bind oxygen as hemoglobin does (6).

The potential of cytochrome is remarkably high in comparison with other iron complexes and even with the hemoglobin-methemoglobin system, which lies 0.1 volt below it (7). This, together with the small amount of pigment present, makes it seem doubtful whether cytochrome can act as a catalyst, except at fairly high potentials, and it remains to be seen whether the catalytic effects heretofore ascribed to cytochrome are due to the pigment itself,

<sup>2</sup>These results are close to the approximate value, +0.280 volt, estimated by Stone and Coulter for the cytochrome C found in certain bacteria (5).

or to the iron complex which has been shown to accompany it. Cytochrome C does, however, provide an indicator of potential within a certain range, which enables one to estimate the actual oxidation potential within an entirely undisturbed and normal cell.

The conclusions that can be drawn from the titration curves are less definite. Certainly it is not cytochrome which is being titrated. Apparently an iron-protein complex is reacting in a not entirely reversible manner, and a reducing effect present in the dialyzed solutions adds to the complexity. A definite poisoning level is maintained and platinum electrodes appear to record the state of oxidation of the iron. The iron-protein complex must be a fairly undissociated one as it is formed when the iron is added as the complex pyrophosphate. It is to be noted that the concentration of iron present in the solutions which are titrated is much lower than that present in the yeast itself.

#### SUMMARY

1. The oxidation-reduction potential of cytochrome has been determined, and shown to be the same in artificially prepared solutions as in the yeast cell; namely,  $+0.260$  volt (referred to the normal hydrogen electrode).

2. Extracted with the cytochrome, is a colorless iron compound, whose apparent mid-point potential at pH 7.0 in the presence of protein is about  $+0.20$  volt. It is estimated that in the purest preparations of cytochrome heretofore obtained, at least two-thirds of the iron present is in the form of this compound, and that the cytochrome iron forms 0.3 per cent or less of the total yeast iron.

3. It has been shown that the presence of protein raises markedly the oxidation potential of the ferrous-ferric system in various buffers, probably because a strong iron-protein complex is formed. The above colorless iron compound present in yeast is such a complex and its potential is the same as that of the artificially prepared iron-protein complex. Moreover, removal of protein from the yeast extracts markedly lowers the potential of the iron compound.

4. A phosphorous compound is associated with the above complex.

5. The biochemical significance of these findings is discussed.

The author expresses here his gratitude to Dr. Charles N. Frey and his assistants at The Fleischmann Laboratories for generous help in preparing large amounts of yeast extract and to Professor J. B. Conant for invaluable and patient advice and assistance.

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## THE EFFECT OF PARATHYROID EXTRACT ON BLOOD MAGNESIUM\*

BY DAVID M. GREENBERG AND MYRTLE A. MACKEY

*(From the Division of Biochemistry, University of California Medical School, Berkeley)*

(Received for publication, September 13, 1932)

A large amount of work exists tending to show that there is a functional relationship between the metabolism of magnesium and calcium in the animal body. This is seen from the work of Mendel and Benedict (1), Mathews and Austin (2), and particularly Bečka (3), to mention only a few writers on this subject.

If there is a metabolic relationship between magnesium and calcium, it would seem plausible that the parathyroid hormone should also have some effect on blood magnesium as well as on the level of the blood calcium. A search of the literature revealed that Scholtz (4) has carried out some experiments on the influence of parathyroid extracts on serum magnesium, and reports there is a small magnesium increase, coming before the rise in calcium occurs.

An examination of Scholtz's data shows that only relatively small increases in calcium were obtained in his experiments, the maximum being but 3.0 mg. per 100 ml. of serum. Also, no control experiments are given to show that the magnesium changes observed may not merely have been the result of the withdrawal of blood involved in taking the samples for analyses. Furthermore, contrary to Scholtz, it is stated by Hogben and Charles (5) that they could detect no change in the magnesium content of the blood of rabbits following injection with parathyroid extracts. As is well known, rabbits are poor subjects for calcium experimentation.

Because of the discrepancies that have been mentioned, we felt it worth while to conduct some further experiments on the

\* This research was aided by grants from the Christine Breon Fund and the National Research Council.

subject. These were carried out on dogs, parathormone<sup>1</sup> being used as the parathyroid extract. Moderate sized dogs, weighing between 10 and 15 kilos, were selected for the work.

Injections of 100 units of parathormone were given subcutaneously in these experiments and blood was drawn from the femoral artery, about 20 ml. of blood being taken for each sample.

The calcium analyses were carried out by Kirk and Schmidt's (6) method, and the magnesium by our hydroxyquinoline method (7).

Several tests were first run to determine the effect of the hemorrhage due to drawing the samples on the blood magnesium. Blood

TABLE I  
*Effect of Drawing Blood Samples on Plasma Magnesium*

	Sample No.	Time of bleeding, hrs. after first sample	Mg per 100 ml. plasma
			<i>mg.</i>
Dog C	1	0	2.80
	2	3.75	2.75
	3	6.50	2.75
	4	12.25	2.71
	5	23.25	2.66
Dog St	1	0	2.33
	2	2.25	2.34
	3	5.75	2.38
	4	10.25	2.36

was drawn in the amounts and the intervals used with the parathormone experiments. The bleeding, as is shown in Table I, had no effect whatever on the magnesium content of the plasma.

The data for the effect of the parathormone are given in the curves shown in Fig. 1. As these curves show, our experiments confirm Scholtz's finding that there is a small increase in the magnesium produced by the injection of the parathyroid extract. The magnesium increase comes very considerably before the increase in calcium manifests itself, taking place between the 2nd and 6th hours after the injection and then quickly dropping back to the initial value. The increase in the plasma magnesium is relatively

<sup>1</sup> The parathormone was generously furnished by Eli Lilly and Company.

small. The average rise in our experiments was 0.6 mg. of magnesium per 100 ml. of plasma and the extreme variation 0.4 to 1.0 mg. of magnesium per 100 ml. of plasma. The magnesium increase, it would appear, is independent of the height subsequently attained by the blood calcium.

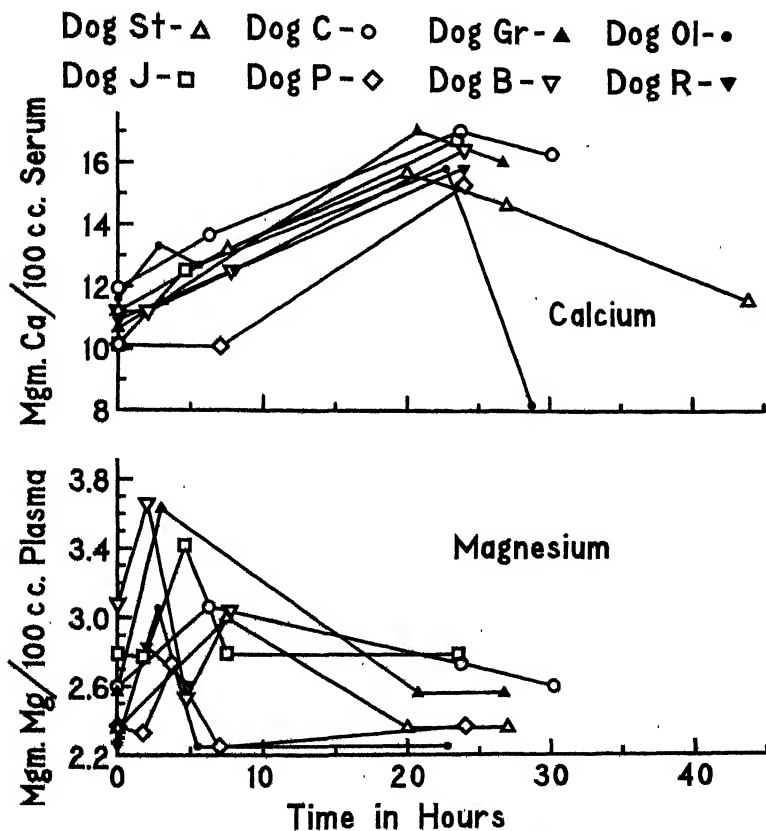


FIG. 1. The effect of parathormone on blood magnesium. Each dog was injected with 100 units of parathormone.

With the slight basis available, it does not seem worth while to speculate on the cause for this effect of parathyroid extract on the magnesium.



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# IRRADIATED MILK: THE INFLUENCE OF THE INTENSITY AND CHARACTER OF THE RADIATIONS ON THE ANTIRACHITIC POTENCY

BY G. C. SUPPLEE

*(From the Research Laboratories of The Dry Milk Company, Bainbridge, New York)*

AND H. H. BECK AND M. J. DORCAS

*(From The National Carbon Company, Inc., Cleveland)*

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The complicated character of the photochemical reactions involved in the synthesis of vitamin D from pure ergosterol and sterol concentrates by ultra-violet rays has been indicated by various spectrographic and analytical studies. It is significant that these investigations, although undertaken with somewhat different objectives in view, have, nevertheless, shown that variable quantities of the radiant energy within the known antirachitic range have a variable effect upon the chemical and biological character of such irradiated products (1-5). While parallel studies have not been conducted extensively with natural food products of heterogeneous composition, such data as are available from milk irradiated under standardized and controlled conditions show that, with certain limitations, the antirachitic potency of this food product bears a certain relationship to the amount of energy applied. Previous papers by the authors have reported data from unscreened energy sources substantiating this relationship (6-8). The present paper contains further data resulting from a continuation of similar studies but wherein lower amounts of energy of different quality were applied to milk under uniform conditions previously described.

## EXPERIMENTAL

The radiations used in this investigation were obtained from carbon arcs of the flaming arc type and quartz mercury vapor arcs

of the Hanovia type. In order to obtain a range of variations in the intensity and quality of the radiations from each source, certain controllable factors were introduced in the experimental plan. The intensity of the direct radiations from each of the sources was varied by changing the amperage of the arc stream while maintaining a constant voltage. A further variation in the intensity was made possible by using reflected rays only, and as obtained from nickel and chromium reflecting surfaces designed to disperse or concentrate the reflected radiations. In order to eliminate the rays that would have been incident on the milk directly from the source, a small asbestos shield was placed close to the arc. This shield intercepted all direct radiations without interfering with the rays normally directed on the milk by the reflectors. Both the character and intensity of the radiations from each source were varied by the use of Corex D and mica filters. The mica filters were of the best grade sheet mica of substantially uniform thickness.

Spectroradiographic measurements (9-11) were made for each of the different sources of radiation unscreened and burning at different emission intensities, from which data spectral energy distribution curves were plotted. These curves were used as a basis for obtaining similar data for the screened radiations by taking into consideration the transmitting properties of the particular screen. The Corex D screen transmits substantially no energy below 2700 Å., approximately 13 per cent at 2800 Å., 33 per cent at 2900 Å., and substantially 60 per cent at 3000 Å. The mica screen transmits practically no energy below 2900 Å. and approximately 15 per cent at 3000 Å. The transmission of the Corex D screen between 3000 and 3150 Å. varies from 60 to 75 per cent, and that of the mica screen from 15 to 45 per cent.

The physical data were used to calculate (6, 7) the energy from 2000 to 3000 Å. in terms of ergs and quanta per second per square mm. of area at a given distance from the arc. From such data the amount of energy applied per cc. of milk, treated under the uniform conditions previously described, was calculated for each condition under which the milk was irradiated. Milk, in 1000 pound lots, was exposed in a thin flowing film for periods varying from 8 to 48 seconds. Samples were taken for biological assay after exposure periods of 8, 16, 32, and 48 seconds. Immediately following irra-

diation, the fluid milk was desiccated by the Just process and hermetically sealed in inert gas.

The vitamin D potency of the experimental samples was determined on the reconstituted dry milk by the curative method previously used (6, 7). Since it was desired to obtain results from these assays which would be comparable with those obtained from previous similar studies, such quantities of milk were fed daily for the 10 day test period as would give data showing the amount of milk required to give a +1 line test, which we have used as a standard for evaluating a uniform minimum detectable antirachitic effect. In accordance with common experience, the individual animals of groups receiving the same milk fed at the same level showed certain variations in degree of calcification. Such variations, however, are not particularly relevant for interpretative purposes if there are a sufficient number of animals per group, and particularly if a number of groups are fed different levels of the same milk. Since all irradiated samples were prepared at the same time from the same general milk supply and were subjected to the same irradiation technique, comparable conditions for determining the effect of the different radiations were assured in so far as possible. The well defined curves indicate a satisfactory degree of accuracy and comparability, and it is considered that the amounts of the different irradiated milks recorded as necessary to produce the +1 degree of calcification under the particular test conditions are correct to within 10 to 15 per cent.

Known amounts of energy per cc. of milk having been applied and the quantity of such milk required to give a uniform antirachitic effect having been determined, basic data are available from which fundamental relationships between antirachitic activity and applied energy may be shown. Table I contains detailed data and the calculated values obtained from this series of experiments. Charts I, II, III, and IV have also been prepared in order to facilitate interpretation. In these charts the calculated number of molecules of vitamin D per cc. of milk have been plotted against the energy in quanta (2000 to 3000 Å.) applied per cc. of milk. The curves have been plotted on logarithmic coordinate paper in order to obtain a more uniform spread of the points and because the data appeared to be a parabolic function which can be more readily interpreted when plotted so as to present a straight line

TABLE I

*Relation of Amount of Energy (2000 to 3000 Å.) Applied to Milk and Vitamin D Concentration*

Group No.	Sample No.	Arc	Exposure period	Total ergs per cc. milk ( $\times 10^9$ )	Total quanta per cc. milk ( $\times 10^{14}$ )	Total milk fed	Vitamin D per cc. $\times 10^{10}$
			sec.			cc.	mols
I-AC-45	1	C carbon	8	921	1,218	45	100
	2		16	1764	2,333	30	150
	3		32	3432	4,540	20	225
	4		48	5360	7,091	15	300
I-AC-60	5	C carbon	8	989	1,323	45	100
	6		16	2016	2,676	20	225
	7		32	4292	5,700	15	300
	8		48	6780	9,012	15	300
I-AC-80	9	C carbon	8	1304	1,725	35	128
	10		16	2836	3,730	17.5	256
	11		32	5748	7,560	10	450
	12		48	9486	12,480	10	450
I-BCD-45	13	C carbon, Corex D	8	110	162	120	37
	14		16	196	288	100	45
	15		32	478	604	65	70
	16		48	759	1,118	50	90
I-BCD-60	17	C carbon, Corex D	8	156	229	100	45
	18		16	298	439	75	60
	19		32	577	849	60	75
	20		48	885	1,303	45	100
I-BCD-80	21	C carbon, Corex D	8	192	282	100	45
	22		16	367	540	70	64
	23		32	691	1,017	55	82
	24		48	1137	1,918	37	121
I-DCM-45	25	C carbon, mica	8	11	16	150	30
	26		16	21	31	150	30
	27		32	44	65	150	30
	28		48	67	99	150	30
I-DCM-60	29	C carbon, mica	8	12	18	150	30
	30		16	23	34	150	30
	31		32	49	72	150	30
	32		48	80	118	150	30
I-DCM-80	33	C carbon, mica	8	17	25	150	30
	34		16	38	56	150	30
	35		32	75	110	150	30
	36		48	112	165	150	30

TABLE I—Continued

Group No.	Sample No.	Arc	Exposure period	Total ergs per cc. milk ( $\times 10^9$ )	Total quanta per cc. milk ( $\times 10^{10}$ )	Total milk fed	Vitamin D per cc. $\times 10^3$
			<i>sec.</i>			<i>cc.</i>	<i>mole</i>
I-ECR-45	37	C carbon, reflected	8	409	545	75	60
	38		16	851	1,134	55	82
	39		32	1549	2,064	32.5	138
	40		48	2228	2,970	30	150
I-ECR-60	41	C carbon, reflected	8	502	669	65	70
	42		16	873	1,163	55	82
	43		32	1580	2,106	32.5	138
	44		48	2441	3,253	22.5	200
I-ECR-80	45	C carbon, reflected	8	592	789	60	75
	46		16	1256	1,674	35	128
	47		32	2439	3,251	22.5	200
	48		48	3819	5,090	15	300
II-AS-45	61	Sunshine carbon	8	201	278	100	45
	62		16	414	573	80	56
	63		32	834	1,155	70	64
	64		48	1288	1,784	60	75
II-AS-60	65	Sunshine carbon	8	252	345	50	90
	66		16	528	762	25	180
	67		32	1018	1,392	20	225
	68		48	1557	2,130	17.5	256
II-AS-80	69	Sunshine carbon	8	339	459	35	128
	70		16	694	938	30	150
	71		32	1412	1,912	18	250
	72		48	2112	2,862	15	300
II-BSD-45	73	Sunshine carbon, Corex D	8	31	46	120	37
	74		16	64	94	120	37
	75		32	128	189	100	45
	76		48	196	290	100	45
II-BSD-60	77	Sunshine carbon, Corex D	8	43	63	120	37
	78		16	85	125	120	37
	79		32	171	253	100	45
	80		48	243	359	100	45
II-BSD-80	81	Sunshine carbon, Corex D	8	42	62	120	37
	82		16	87	128	120	37
	83		32	181	267	100	45
	84		48	279	413	90	50
II-DSM-45	85	Sunshine carbon, mica	8	3	4	150	30
	86		16	6	8	150	30
	87		32	12	18	150	30
	88		48	20	29	150	30

TABLE I—Continued

Group No.	Sample No.	Are	Exposure period	Total eggs per cc. milk ( $\times 10^6$ )	Total quanta per cc. milk ( $\times 10^6$ )	Total milk fed	Vitamin D per cc. $\times 10^6$
			sec.			cc.	mols
II-DSM-60	89	Sunshine carbon,	8	4	6	150	30
	90	mica	16	8	12	150	30
	91		32	16	23	150	30
	92		48	25	37	150	30
II-DSM-80	93	Sunshine carbon,	8	6	8	150	30
	94	mica	16	13	21	150	30
	95		32	24	35	150	30
	96		48	36	53	150	30
II-ESR-45	97	Sunshine carbon,	8	76	105	120	37
	98	reflected	16	151	209	100	45
	99		32	267	370	100	45
	100		48	413	573	90	50
II-ESR-60	101	Sunshine carbon,	8	95	131	120	37
	102	reflected	16	180	249	100	45
	103		32	384	532	90	50
	104		48	614	851	80	56
II-ESR-80	105	Sunshine carbon,	8	179	248	100	45
	106	reflected	16	316	438	90	50
	107		32	595	825	80	56
	108		48	920	1,276	70	64
III-AMg-45	121	Magnesium car-	8	413	576	45	100
	122	bon	16	890	1,241	27.5	164
	123		32	1683	2,347	20	225
	124		48	2759	3,849	15	300
III-AMg-60	125	Magnesium car-	8	796	1,105	30	150
	126	bon	16	1548	2,150	25	180
	127		32	3124	4,340	17.5	257
	128		48	4740	6,588	12.5	360
III-AMg-80	129	Magnesium car-	8	1225	1,700	17.5	287
	130	bon	16	2430	3,374	15	300
	131		32	4740	6,580	10	450
	132		48	7670	10,620	10	450
III-BMgD-45	133	Magnesium car-	8	69	100	80	56
	134	bon, Corex D	16	139	202	80	56
	135		32	287	417	65	70
	136		48	352	513	65	70
III-BMgD-60	137	Magnesium car-	8	106	154	80	56
	138	bon, Corex D	16	219	319	65	70
	139		32	450	655	60	75
	140		48	677	986	50	90

TABLE I—Continued

Group No.	Sample No.	Arc	Exposure period	Total ergs per cc. milk ( $\times$ $10^6$ )	Total quanta per cc. milk ( $\times 10^{10}$ )	Total milk fed	Vitamin D per cc. $\times 10^3$
			sec.			cc.	mole
III-BMgD-80	141	Magnesium car- bon, Corex D	8	148	215	70	65
	142		16	242	352	65	70
	143		32	587	855	55	82
	144		48	968	1,410	45	100
III-DMgM-45	145	Magnesium car- bon, mica	8	3	5	150	30
	146		16	6	9	150	30
	147		32	11	16	150	30
	148		48	17	25	150	30
III-DMgM-60	149	Magnesium car- bon, mica	8	4	6	150	30
	150		16	11	16	150	30
	151		32	18	26	150	30
	152		48	28	41	150	30
III-DMgM-80	153	Magnesium car- bon, mica	8	16	23	150	30
	154		16	32	47	150	30
	155		32	61	90	150	30
	156		48	95	140	150	30
III-EMgR-45	157	Magnesium car- bon reflected	8	170	236	67	67
	158		16	343	476	60	75
	159		32	742	1,031	32	141
	160		48	1069	1,485	25	180
III-EMgR-60	161	Magnesium car- bon, reflected	8	276	383	57	79
	162		16	581	807	37	121
	163		32	1166	1,620	25	180
	164		48	1741	2,420	20	225
III-EMgR-80	165	Magnesium car- bon, reflected	8	494	686	42	107
	166		16	967	1,344	25	180
	167		32	1962	2,737	20	225
	168		48	2987	4,152	17.5	256
IV-AHg	181	Mercury vapor	8	342	459	30	150
	182		16	650	870	26.7	168
	183		32	1328	1,784	23.8	198
	184		48	1938	2,598	20	225
IV-AHgCr	185	Mercury vapor	8	121	163	120	37
	186		16	236	314	100	45
	187		32	464	625	90	50
	188		48	708	951	80	56
IV-BHgD	189	Mercury vapor, Corex D	8	46	68	100	45
	190		16	93	137	75	60
	191		32	185	273	60	75
	192		48	278	410	50	90



TABLE I—*Concluded*

Group No.	Sample No.	Arc	Exposure period	Total ergs per cc. milk ( $\times 10^9$ )	Total quanta per cc. milk ( $\times 10^{14}$ )	Total milk fed cc.	Vitamin D per cc. $\times 10^4$ mols
IV-BHgDCr	193	Mercury vapor,	8 sec.	14	20	120	37
	194	Corex D	16	27	40	120	37
	195		32	56	82	120	37
	196		48	86	127	120	37
IV-DHgM	197	Mercury vapor,	8	6	8	150	30
	198	mica	16	11	16	150	30
	199		32	23	38	150	30
	200		48	35	56	150	30
IV-DHgMCr	201	Mercury vapor,	8	2	3	150	30
	202	mica	16	4	6	150	30
	203		32	7	10	150	30
	204		48	10	14	150	30
IV-EHgR	205	Mercury vapor,	8	120	161	80	56
	206	reflected	16	224	300	50	90
	207		32	443	594	35	128
	208		48	649	870	25	180
IV-EHgRCr	209	Mercury vapor,	8	27	36	120	37
	210	reflected	16	54	72	100	45
	211		32	117	158	100	45
	212		48	184	249	90	50
IV-EHgM	224	Mercury vapor,	80	(31)	90	100	45
IV-DHgMCr	237	" "	160	226	334	120	37

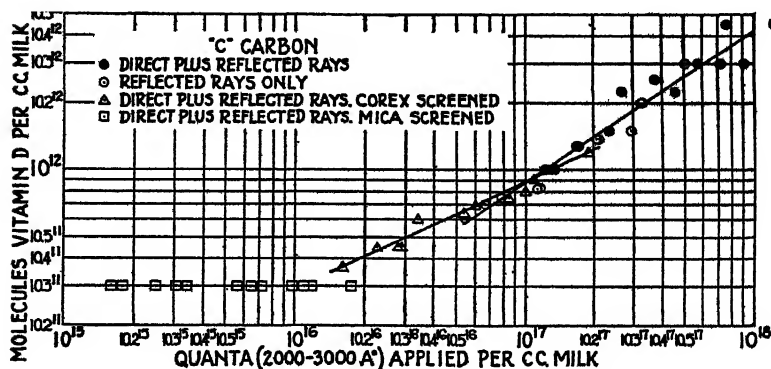


CHART I. Antirachitic activity and applied energy relationships

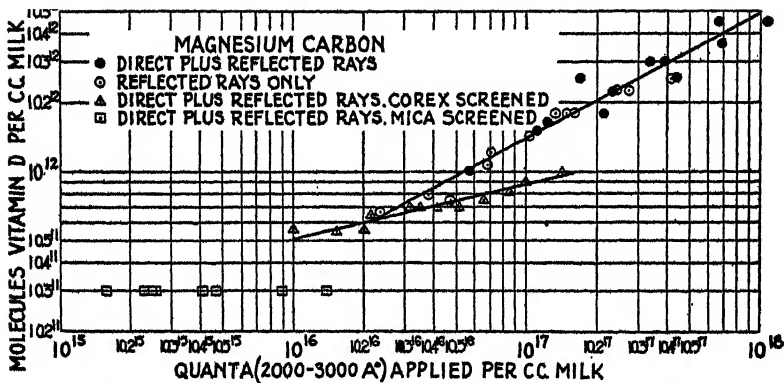


CHART II. Antirachitic activity and applied energy relationships

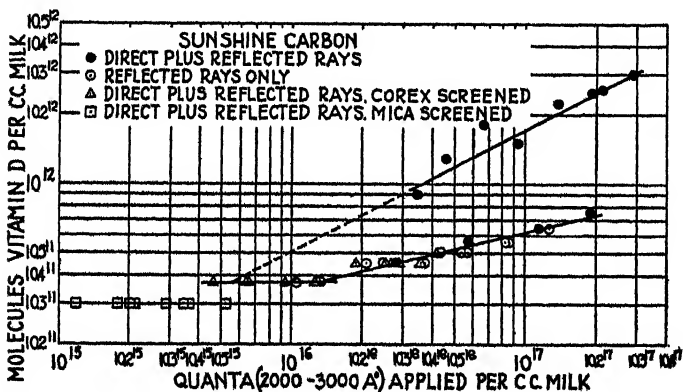
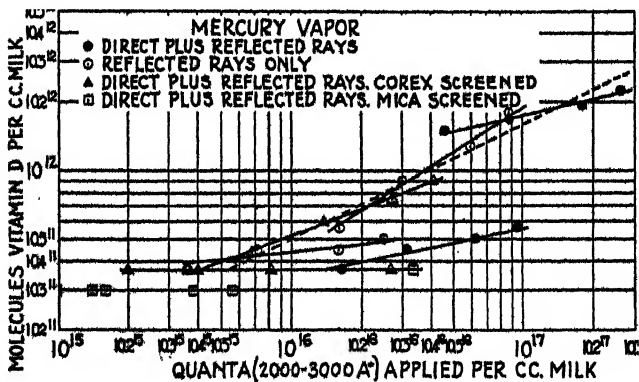


CHART III. Antirachitic activity and applied energy relationships



than when in the form of curves with constantly changing slopes.

In analyzing the data, the manner in which the antirachitic activity varies with the applied energy is of equal importance to the total activity obtained when the properties of various energy sources are considered. Since the slope of the line obtained on logarithmic coordinates determines the power of the argument of the function, equal slopes indicate functions that vary according to similar laws; consequently they represent similar characteristics. Variations in slopes and total values will therefore indicate variations in the activated milk which are characteristic of the various energy sources used.

#### DISCUSSION

The data clearly show the existence of certain accepted relationships between applied energy and the antirachitic potency of the milk; however, other relationships are indicated which have not been apparent heretofore.

Within a given range, successive exposures to energy as applied under the conditions of these tests result in an increase in antirachitic potency of the milk. This relationship generally holds true irrespective of whether the energy was from screened or unscreened arcs, or reflected radiation only. It may be considered, therefore, that within certain limits the antirachitic potency of irradiated milk is dependent upon the amount of energy applied, provided, of course, the radiations are of sufficient intensity and within the known antirachitic range.

The data also show that the character of the radiations affects the rate of increase in antirachitic properties, and that the effectiveness and efficiency of certain energy sources may be further altered by the rate at which the effective energy is applied.

The data for the "C" carbon show a close conformity to a definite law in producing incremental increases in the antirachitic potency of the milk between an energy range of  $409 \times 10^8$  ergs ( $545 \times 10^{14}$  quanta) and  $9486 \times 10^8$  ergs ( $12,480 \times 10^{14}$  quanta). This conformity within these limits does not appear to be affected by varying the intensity of the unscreened arc, or by the intensity of reflected rays only, and is not greatly changed by the filtered rays transmitted by the Corex D screen. The flattening of the curve in the lowest range of applied energy as obtained by the

mica screen can be accounted for only by the assumption that radiations of such character and of such low intensity are ineffective for measurably increasing the vitamin D potency of the milk assayed by the biological method used. A similar flattening of the curve in the region of the highest applied energy would apparently be due to completion of the reactions involved, as pointed out in a previous paper (7), resulting from either exhaustion of the substance which can be activated or the influence of secondary or counter reactions destructive to the vitamin.

The data from the magnesium carbon when similarly analyzed show general relationships analogous to those shown by the C carbon, in that the data for screened and unscreened energy lie along separate curves whose slopes are not greatly less than those of the C carbon. It will be observed, however, that for the same or equivalent amounts of energy applied from the two sources, the degree of effectiveness of the radiations from the magnesium carbon is greater than those from the C carbon. This relationship seems to be true irrespective of whether direct, reflected, or screened rays are involved. The qualitative character of the radiations from the two sources no doubt explains, in part at least, the difference in effectiveness of the energy. It seems apparent that the lower rate, at which the energy from the Corex D-screened magnesium arc is applied, is a factor which tends to lower the effectiveness of such energy, and also to alter the rate at which vitamin D is formed. It is significant that a similar degree of change in effectiveness of the energy from the screened C carbon is not apparent.

In considering the data from the sunshine carbon, certain relationships analogous to those found in the C and magnesium carbons are to be noted. However, there are indications of other phenomena not clearly shown by the results from these latter sources. There is a similarity between the results from the different sources at the higher energy levels, and also between the sunshine and magnesium arc sources, in that these results diverge into two curves of markedly different slope. This divergence is very marked in the case of the sunshine carbon and takes place in a considerably lower energy range. In the case of the magnesium carbon only the screened energy data lie in the lower curve, while in the case of the sunshine carbon, not only the screened energy results, but

also all results from the reflected energy and those from the direct radiations (45 ampere operation) of lower intensity.

An analysis of the various results showing differences in the proportional relationships as exhibited by each of the sources functioning at different intensities, not only suggests a very definite relationship between the quality, quantity, and intensity of the building energy, and the degree of antirachitic potency imparted to the milk, but also leads to the pertinent conclusion that the intensity of the energy applied for the irradiation of milk is a matter of considerable importance in determining the degree of potency attained, and irrespective of the quality of the radiation.

The results from the mercury vapor arcs show a greater similarity to those of the sunshine carbon arcs than they do to those of the C or magnesium carbon arcs. The divergence of the curves at the lower energy ranges is pronounced in the mercury vapor arc results, and in similarity with the sunshine carbon results, the divergence is not caused by the screened radiations only, but rather by intensity of the applied energy.

The conformity of the data from all sources, and particularly at the higher energy levels, to slopes of similar character indicates close adherence to a common law, at least for practically all unscreened sources, between an energy range from approximately  $6 \times 10^{16}$  quanta to  $5 \times 10^{17}$  quanta per cc. of milk. However, the effectiveness of the energy between these limits for increasing the antirachitic potency of the milk varied with the different radiations even when the same or equivalent amounts of energy were applied from different sources. The relative effectiveness of an equivalent amount of energy from the different sources was found to be in the following order: C carbon, magnesium carbon, mercury vapor arc, and sunshine carbon, respectively. It should be noted, however, that when time and intensity are considered for practical purposes, the effectiveness of the different sources increases in the following order: mercury vapor arc, sunshine carbon arc, magnesium carbon arc, and C carbon arc.

The data clearly show that the effect of using Corex D-filtered radiations is to reduce the amount of energy reaching the milk per unit of time, thus causing a commensurate reduction in antirachitic potency produced within a given period as compared with unscreened radiations. Of the sources of energy used, an appreci-

able effect resulting from the filtering out of the shorter wavelengths was noted only in the case of the magnesium carbon, and this effect was no doubt due to the particular character of the radiations from this source.

Mica screens which transmit substantially no energy below 2900 Å. and only 45 per cent at 3150 Å. did not transmit sufficient radiation from any of the sources to cause any significant or measurable antirachitic properties in the milk treated under the conditions of these experiments.

The reduction in the efficiency and effectiveness of the radiations at the lowest energy levels may be due to one or more causes, such as lack of sufficient energy to initiate the reaction, an initial degree of absorption by substances which cannot be activated, or possibly to reflection of a substantial proportion of the incident rays. Reduction in efficiency and effectiveness at the extremely high levels of applied energy is no doubt due to the approach of completion of the reaction, or possibly the dominance of secondary reactions detrimental to the potency of the vitamin.

#### SUMMARY AND CONCLUSIONS

1. The total radiations from unscreened sources used in these experiments produced a higher antirachitic potency in the milk than the filtered radiations. The filtered radiations reduced the total energy applied per unit of time and caused a commensurate reduction in the antirachitic potency of the milk.

2. A definite intensity of radiation within the antirachitic range is necessary for effective and efficient utilization of energy for the antirachitic activation of milk.

3. The progressive increase in the antirachitic potency of milk during irradiation follows definite principles within certain limits of applied energy irrespective of the source of radiation used. The rate of development of the antirachitic potency within these limits is, however, influenced by the qualitative character and intensity of the energy applied.

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## THE BASIC AMINO ACIDS OF LIVETIN

By T. H. JUKES\* AND H. D. KAY

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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In view of the prominence of the egg protein livetin as a constituent of food, it is unfortunate that so little is known as to its amino acid content. Kay and Marshall (1) have obtained values for its tyrosine, tryptophane, and cystine content. It is the purpose of this communication to present data concerning the amounts of the amino acids, arginine, lysine, and histidine in livetin.

### *Preparation of Livetin*

Hen's egg yolks were washed as described by Kay and Marshall (1), the contents were mixed with an equal volume of 8 per cent sodium chloride, and the solution of the mixed proteins (lecithovitellin and livetin) was shaken with five or six changes of ether. The resulting opalescent solution was dialyzed in collodion bags against running tap water for from 24 to 36 hours. (A satisfactory collodion was obtained by dissolving 5 gm. of pyroxylin (J. T. Baker) in 10 cc. of ethylene glycol, 25 cc. of absolute alcohol, and 65 cc. of ether (2).) At the end of this time the lecithovitellin had precipitated, the livetin remaining in solution. The mixture was centrifuged and the precipitate washed once with water. The separated solutions were combined and clarified by passing through paper pulp; one-half of the volume of 95 per cent alcohol was added, the mixture brought to pH of about 5.0 with HCl, and boiled under a reflux condenser for several hours. The coagulated livetin was then filtered off and extracted in a Soxhlet apparatus with hot 95 per cent alcohol for 10 to 20 hours, then

\* Ontario Research Foundation Fellow,



transferred to another Soxhlet apparatus containing ether over calcium chloride where extraction was continued for about 30 hours. The protein was removed to a dish and the ether allowed to evaporate. Without further drying, the livetin was powdered and passed through a 100 mesh sieve. When dried at 110° it contained 14.8 per cent of nitrogen and 0.54 per cent of ash. Only about 0.1 per cent of phosphorus was usually present, indicating the absence of appreciable quantities of vitellin.

#### *Determination of Arginine by Arginase Method*

The technique of Hunter and Dauphinee (3) was employed with the following modifications and additions. (1) After determination of amide nitrogen and separation of the hydrolysate from the alkali-insoluble humin, the filtrate was acidified with sulfuric acid (instead of hydrochloric). The resultant precipitate of calcium sulfate was centrifuged off. 200 cc. of water were added to the precipitate. The mixture was boiled down to small bulk and centrifuged. This was repeated six times. The combined washings were evaporated with the main hydrolysate and made up to volume (usually 50 cc.). (2) Antisplash bulbs (air filters) in aeration tubes were filled with glass wool and glass beads, washed in a current of water, and dried at 120° after each determination. (3) Receiver tubes were thoroughly shaken, when the end-point was reached in titration, to dissolve out traces of acid in the capryl alcohol. If necessary, further alkali was added to complete the titration. (4) To insure high potency, crude arginase preparations were made from (beef) liver of an animal killed on the same day. The preparations were never used after they were more than 4 days old. (5) Arginase blanks were determined on aliquots of the crude arginase, diluted, and buffered in the same way as the actual digests. The blanks were allowed to stand under the same conditions. (6) A deaminase blank was used in preference to an amide blank; this is a more satisfactory procedure and is just as accurate if the arginase blank is low (*i.e.*, if the arginase preparation is fresh).

Determinations were made upon hydrolysates of two different preparations, Livetin A and Livetin B. A sample of vitellin, washed free from livetin and freed from bound lipids by prolonged

extraction with boiling alcohol (Vitellin 1)<sup>1</sup> was examined under the same conditions, arginine determinations being carried out upon it with two different enzyme preparations (Arginase A and Arginase B). "Principal" tubes always were run in triplicate, others in duplicate. The results are recorded in Table I.

Differences between the two proteins make themselves apparent: the humin nitrogen figure is 2.14 per cent, as against 1.36 per cent for vitellin, corresponding to the higher tryptophane content of livetin (1). Furthermore, livetin contains 11.7 per cent of its nitrogen as arginine; vitellin contains 14.7 per cent.

TABLE I  
*Analysis of Livetin and Vitellin by Arginase Method*

	Livetin A with Argi- nase A	Livetin B with Argi- nase B	Vitellin 1 with	
			Argi- nase A	Argi- nase B
Amide N, per cent.....	8.17	8.37	8.38	
Humin " (sum of both fractions), per cent	2.12	2.15	1.36	
Volume of hydrolysate for digests, cc.....	5	5	5	5
Protein N corresponding, mg.N.....	86.0	54.3	58.5	58.5
Gross result, mg.N.....	5.98	3.88	4.92	5.05
Net total blank, mg.N.....	1.01	0.78	0.70	0.79
Urea N from arginine, mg.N.....	4.97	3.10	4.22	4.26
Arginine N + 1.5 per cent, mg.N.....	10.1	6.30	8.56	8.66
" " per cent of total N.....	11.75	11.60	14.63	14.80

*Determination of Cystine by Method of Sullivan (5)*

Criticisms of Folin's colorimetric technique for cystine determination as giving too high values have recently appeared (5-7). As figures for the cystine content of vitellin and livetin by the naphthoquinone method of Sullivan (5) have not yet appeared, they are published herewith. Difficulty was experienced in obtaining very good duplicates either with standard cystine solutions or with protein hydrolysates by this method. Darco charcoal (Eastman Kodak Company) was found more efficient than several other brands tried, including that mentioned by

<sup>1</sup> Vitellin was found to contain 3.8 per cent of ash. Calvery and White (4) found only 0.32 per cent of ash, which seems hardly compatible with their figure for phosphorus (0.92 per cent).

Sullivan. Vitellin is a difficult protein to deal with, probably owing to its high content of iron and phosphorus; livetin gives a much more satisfactory color.

The method gave 2.3 per cent of cystine for livetin and 1.1 per cent for vitellin, while the per cent of total nitrogen as cystine nitrogen was 1.8 for livetin and 0.85 for vitellin. These figures, which represent the mean of a considerable number of determinations, are distinctly lower than those of Kay and Marshall (1), who used the method of Folin and Looney (8) and found 3.9 per cent of cystine in livetin, and 1.4 per cent in vitellin.

#### *Determination of Bases by Isolation and Weighing As Salts*

Colorimetric methods for determination of the amino acids are subject to question and revision, and the method of Van Slyke for basic nitrogen distribution has also received its share of criticism. Isolation from a protein hydrolysate of the salt of an amino acid, however, gives a figure which cannot be too high. Accordingly, it was decided to employ the method of Kossel and Kutscher (9) for determination of basic amino acids, as revised by Vickery and Leavenworth (10), advantage being taken of the modifications proposed by Calvery (11) for dealing with small amounts of protein. In spite of the recent contention of Rosedale and da Silva (12) that arginine and histidine are not separated under the conditions specified, we found that by stirring at pH 7.4 for 1 hour and allowing the material to stand overnight the histidine silver separated well from the arginine fraction. In the case of livetin, however, the yield of arginine is poor, and the lysine fraction is grossly impure. It was found essential to work up the lysine residues for arginine. Considerable nitrogen loss occurs in the numerous rejected precipitates during the course of the separation. These were examined by the Kjeldahl method, and nitrogen loss was found to be reduced by the following means. (1) All precipitates were boiled with the appropriate wash mixture, then dried at 110–120°, powdered, and rewashed. This was repeated two or three times. (2) Certain precipitates lent themselves also to re-solution and reprecipitation. This was found to reduce nitrogen loss, particularly in the case of the barium phosphotungstate in the purification of lysine, which was treated alternately with sulfuric acid and barium hydroxide.

The results given in Table II represent the best figures obtained from three livetin hydrolysates, each representing from 5 to 7 gm. of protein. They are compared with figures simultaneously obtained for Vitellin 1, and with values reported by other investigators who used the same method. The figures represent percentage of total nitrogen calculated from arginine and histidine flavianates and recrystallized lysine picrate.

TABLE II  
*Basic Nitrogen Distribution of Livetin and Vitellin*

Protein	Arginine N	Histidine N	Lysine N
	<i>per cent of total N</i>	<i>per cent of total N</i>	<i>per cent of total N</i>
Livetin.....	10.3	2.11	5.92
Vitellin.....	13.5	2.07	6.38
“ (Calvery and White (4))*.....	16.63	2.20	6.87
“ (Osborne and Jones (13)).....	15.5	3.3	5.95

\*Recalculated from Table I of the article by Calvery and White.

#### DISCUSSION

The arginase method for the determination of arginine in proteins is free from the inevitable mother liquor losses that occur in isolation methods for the determination of amino acids, and free from the vagaries that attend colorimetry.

In the case of vitellin, we were able to obtain figures for arginine flavianate that corresponded better with the results of the arginase method than in the case of livetin. The figures we give for lysine are probably low, owing to the difficulties experienced in purifying the lysine fraction.

#### SUMMARY

1. As determined by the arginase method, livetin contained 11.7 per cent of its nitrogen in the form of arginine nitrogen. The corresponding value for vitellin was 14.7 per cent.

2. Livetin contained 2.3 per cent of cystine by the Sullivan method, while vitellin contained 1.1 per cent.

3. Isolation of the basic amino acids of livetin gave a nitrogen distribution of 10.3 per cent arginine N, 2.11 per cent histidine N, and 5.92 per cent lysine N.

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